Chapter 3

Initial Studies With Goldfish

3.1 Introduction

The feeding preferences of sea snakes (Heatwole, 1987) vary from prey specific feeders, to generalized ones like *Aipysurus laevis* of the present study. Since snake venom is thought to have evolved in a feeding context (Section 1.1) this study focuses on the effects of *A. laevis* venom on its prey.

Much as white mice and white rats have become standard animals for a wide range of experimental studies, and serve as a base for comparison with other terrestrial mammals, the goldfish *Carassius auratus* has been used as a laboratory standard for fish studies (Carey and Wright, 1961; Berman, 1981). It is robust and the procedures for manitaining it under laboratory conditions are well known (Coffey, 1977; Faure, 1977).

The purpose of this initial study was:

1. to establish a standard animal to be used as a comparative standard for further studies.

2. to develop experimental techniques to be used with the marine prey species.

3. to define signs that could be further examined with the marine prey species.

3.2 Materials and Methods

Forty *C. auratus*, approximately 7 cm in length, were obtained from the Tamworth Pet Centre. They were maintained at the University of New England in all-glass aquaria 63.0 cm x 41.5 cm x 41.0 cm with glass tops. Aeration was provided by two airstones, and Marine Land Maxiflow filters were used to filter the water (Coffey, 1977). All fish were kept (Faure, 1977) for one hour in plastic bags before being released into the system. Daily maintenance of the aquaria included checking air flow and filters and feeding the fish with Tetra Min Staple Fish Food.

The experimental subjects were held in all-glass aquaria 21.0 cm x 15.1 cm x 15.4 cm with glass tops and airstones providing aeration. The fish were allowed to familiarize themselves with the system prior to experimentation.

Injection techniques and dosage calculations were carried out as described in section 2.2 except that the fish were kept in fresh water instead of salt water.

Fish were randomly assigned to one of the following groups:

1. uninjected control group
2. 0.09% saline-injected control group
3. venom-injected group

For each group general behavioural observations were recorded as well as the ventilation rates and time to death, where relevant.

The sternohyoideus muscle (Fig. 2.14) was choosen for examination of myotoxic effects. The selection was based on it's major role in fish gill-ventilation (Section 1.3), and being large enough for easy study.

Removal and processing of the muscle for electron microscopic examination were carried out according to procedures described in section 2.7.

3.3 Results

Envenomated fish demonstrated a common sequence of behavioural changes, which resulted in death in the more serious cases. Death was determined to be at the

time when there was no longer a response to stimulus and no movements occurred. The initial behavioural changes observed in *C. auratus* after envenomation were the presence of erratic movements, an increase in fin movement and greater movement around the aquaria. An increase in ventilation rate (Fig. 3.1) was accompanied by an increase in amplitude of the movements of the mouth and opercular shields. Difficulties in closing the mouth were also noted at this time. Eventually swimming became more erratic and less cordinated, followed by the subject settling to the bottom with an even further elevated ventilation rate. Next came loss of control of the fins, starting with the pectoral fins and continuing back to include the caudal fin. Sporadic uncoordinated swimming followed with loss of buoyancy control in some individuals, and in inability to keep in an upright position in others. In the more advanced stages the fish laid on their sides and showed a steady decrease in ventilation rates.

The observed increase in ventilation first took the form of an increase in ventilatory frequency (buccal and opercular strokes/min), followed by an increase in ventilatory stroke volume (volume of water intake/min), as described in section 1.3. During failure of the ventilatory system, the mouth was the first to cease functioning; it remained open in an extended position. Next the opercular shields stopped moving and also remained extended, leaving the opercular fringe as the last component to cease functioning. With increasing time after envenomation there was a progressive increase in both the ventilatory frequency and ventilatory stroke volume. Eventually, as seen in figure 3.1, the ventilatory frequency then decreased again during advanced stages of envenomation, leaving the subjects with only the increased ventilatory stroke volume, which lasted a while longer. Once the stroke volume decreased only sporadic ventilatory movements were observed. The opercular fringe, which is located on the outer edge of the opercular shields, remained moving a while after cessation of shield movements, but due to its inability to bring enough water to the gills, death shortly followed. There were some recoveries of envenomated fish, but never in cases of complete cessation of ventilation.

Clear to milky-white fecal material was excreted by envenomated individuals; it remained attached to the fish and became extremely long in many cases. Bulging

eyes were also observed in some individuals during the more advanced stages of envenomation (Appendix F).

Envenomated fish did not appear to experience any discomfort. There was no internal hemorrhage observed at autopsy, the heart continued beating long after other vital signs had ceased.

Both the uninjected and saline-injected controls initally demonstrated rapid ventilation followed by a decrease and a leveling off of the rates (Fig. 3.1). There were no observed changes in swimming movements, ventilation rates or any other of the changes that occurred in envenomated individuals. Once the controls became familiar with their new surroundings, they spent the majority of their time foraging on the bottom for food. This foraging activity was rarely observed in the envenomated fish and then only during the earlier stages of envenomation.

Examination of the electron micrographs for the two control groups (Figs. 3.2 and 3.3), revealed only normal muscle with distinct striations. The banding follows the typical patterns of Z lines and accompanying triads of the sarcoplasmic reticulum, as well as distinct A bands, H bands, I bands, M lines and the clearly distinguishable myofilaments, all of which are explained in detail in section 1.2. The micrograph for the envenomated group (Fig. 3.4) shows swelling in the terminal cisternae of the sarcoplasmic reticulum giving a vacuole-like appearance (Stringer *et al.*, 1971). Cloudy swelling, a condition of generalized localized swelling of the sarcomere, can be seen in the micrographs of envenomated fish and is a sign of early pathological change. Hyaline degeneration, a breakdown of the lattice structure of the muscle fiber, is also observable as a homogeneous display (Yokote, 1982), with a disruption of the banding seen in normal muscle tissue (Stringer *et al.*, 1971). These conditions were not observed in the control groups.

3.4 Discussion

The initial studies on goldfish proved valuable in providing experience in fish handling and injection procedures, to later be used on the prey species. Techniques were developed that minimized fish injury and stress, and effiency of the injection procedures

was increased. The experiments with *C. auratus* also provided insight into onset of signs useful in assessing the effects of venom action. Besides LD50 determinations (Finney, 1971), ventilation rates, behavioural observations and electron microscopic examinations were considered to be other fruitful lines of research.

Intramuscular (subcutaneous) injections resemble natural snake bite more closely than other injection techniques (Harris *et al.*, 1975) and are absorbed about as rapidly due to the small molecular size of the hydrophiid venom (Christensen, 1968). Consequently they were employed for the present study. Injections were administered according to the fish's body weight as described in section 2.2. The injection and handling, as well the holding facilities caused no observable problems with the subjects, as demonstrated by comparable ventilation rates (Fig. 3.1), ultrastructural examinations (Figs. 3.2 and 3.3) and behavioural observations of the uninjected and saline-injected controls. Also noted was the absence of hemmorrhage in the injection site in all groups of fish.

Being curare-like in action (Tamiya *et al.*, 1967; Barme, 1968; Lee, 1970; Lee, 1971a; Lee, 1971b; del Castillo and Anderson, 1974; Mebs, 1978; Lee and Lee, 1979) hydrophiid venom is known to have a paralyzing effect on its victims, as described in section 1.1. The orderly shutdown of the ventilatory mechanism of the experimental subjects as the result of envenomation, as well as the loss of fin control with an eventual loss of all movements, due to paralysis, clearly demonstrated a consistent pattern of behavioural changes. Similar sequences of events, as the result of hydrophiid envenomation, were reported in humans (Halstead, 1970; Pickwell, 1972; Reid, 1975) and in laboratory animals (Limpus, 1978; Mebs, 1978; Chang, 1979). Campbell (1979) and Chang (1979) also reported paralysis in an orderly fashion in experimental animals involving the muscles of the head, those involved in swallowing and the eye muscles first, followed by the muscles of limbs and trunk, with eventual impairment of peripheral nerve activity and neuromuscular transmission servicing the respiratory system (Gitter and deVries, 1967; Lee, 1971a) and resulting in respiratory failure.

The initial increase in erratic movements, was possibly the result of changes perceived by the fish as the result of envenomation. The loss of fin control from an anterior to a posterior direction created an increasing problem with swimming, as

well as the ability to control body positioning and movements (Alexander, 1967). During later stages of envenomation there was a total loss of all controllable movements, with only sporadic movements remaining. This complete paralysis could serve an important function of quieting a potentially struggling prey, thus preventing the fish from escaping or causing jaw, buccal or other damage to the snake (Minton, 1974; Heatwole, 1977; Gans, 1978; Sutherland, 1983). The sporadic movements appeared to be an attempt by the fish to regain control of coordinated movements, as well as being typical spasms seen in envenomated subjects during later stages of envenomation (Reid, 1956; Limpus, 1978). The increase in ventilatory frequency and ventilatory stroke volume, may have been an attempt by the fish to compensate for an hypoxic condition (Moyle and Cech, 1982) brought about by envenomation. The increase in the stroke volume is defined by the appearance of pronounced and exaggerated movements of the mouth and opercular shields, and can be explained by an increase in the muscular activity, as well as addition of other head muscles to the process (Ballintijn and Hughes, 1965). Both the elevated ventilatory frequency and the increase in ventilatory stroke volume bring more water into the buccal cavity through quicker and stronger pumping action, respectively.

As discussed in section 1.1, the presence of neurotoxins in snake venom has been well documented, with the effect of myotoxic substances also being reported. Muscular necrosis as the result of sea snake envenomation, however, has not been well documented, with evidence of it observed only in humans (Reid, 1956; Marsden and Reid, 1961; Reid, 1961; Barme, 1968; Halstead, 1970; Reid, 1975; Mebs, 1978; Chang, 1979; Reid, 1979; Mebs and Samejrma, 1980; Minton and Minton, 1980) as a result of medical interest in bite victims, and in a few other higher vertebrates (Carey and Wright, 1961; Geh and Toh, 1976; Fohlman and Eaker, 1977; Brook *et al.*, 1987). The lack of information regarding the myotoxic effects of sea snake venom, especially on lower vertebrates, warranted a search for it in the muscle tissue of fish.

Variation from the normal muscle morphology (Section 1.2) (Figs. 3.2 and 3.3), according to Yokote (1982), reflects changes in the sarcoplasm and is a clear sign of a pathological condition. Bone (1978) and Yokote (1982) point out, that there has been insufficient research and experimental pathology carried out with fish muscle.

Pathological changes in the muscle of teleosts, however, are presently considered to be the same as higher vertebrates and are treated accordingly in this study.

The cloudy swelling, hyaline degeneration (Yokote, 1982), abnormal banding and swollen sarcoplasmic reticulum (Stringer *et al.*, 1971; Tu, 1977) observed in this study (Fig. 3.4) are signs of early pathological change. These findings give possible evidence of pathological changes as the result of myotoxic components in the venom of *A. laevis*. They are only indications of early pathological changes, however, since, due to the high dosage of the venom, only a short time of envenomation occurred before the fish were sacrificed. With the marine prey species, lower dosages of venom permitted longer envenomation times, which in turn allowed for later stages of pathological changes to be observed. This is discussed in a later chapter.

The presence of white fecal material, as well as the bulging eyes, are examples of other envenomation effects which will be examined in more detail for the marine fish.

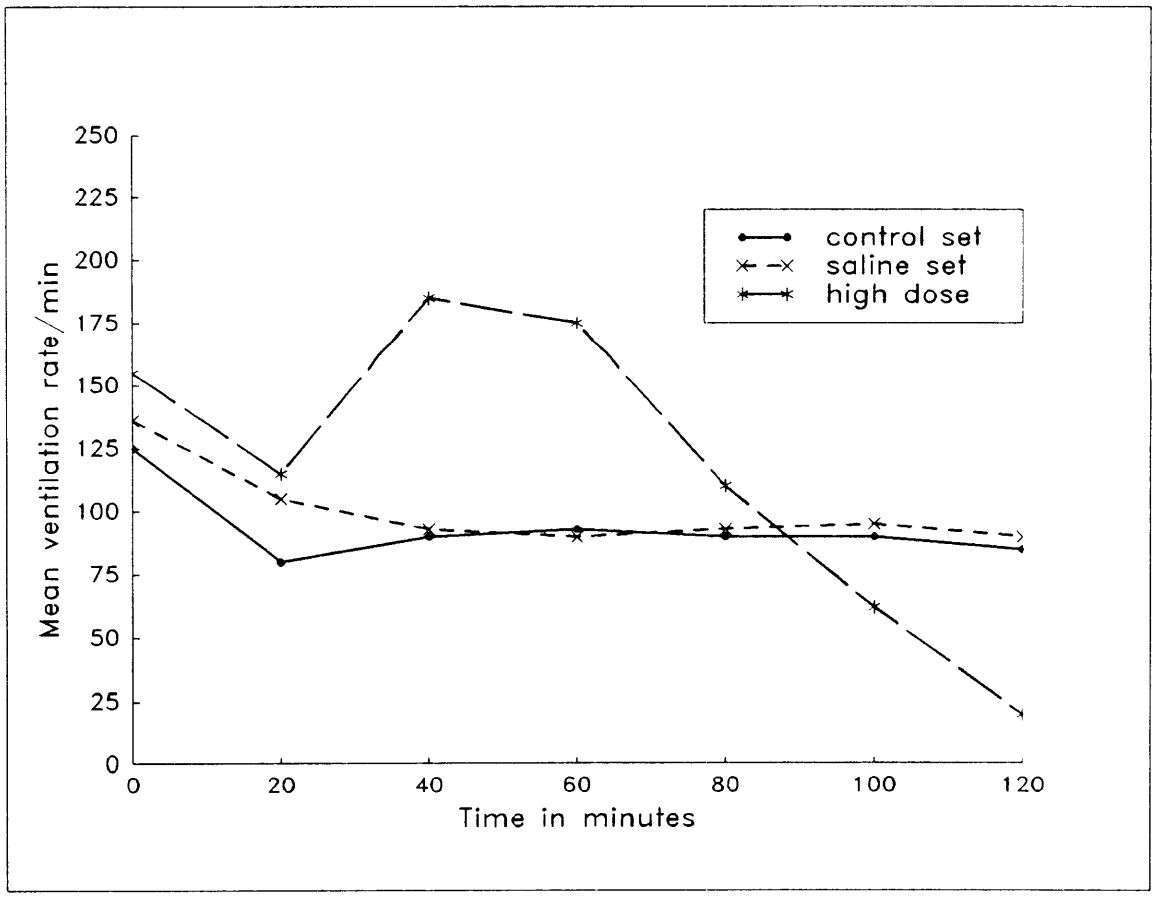
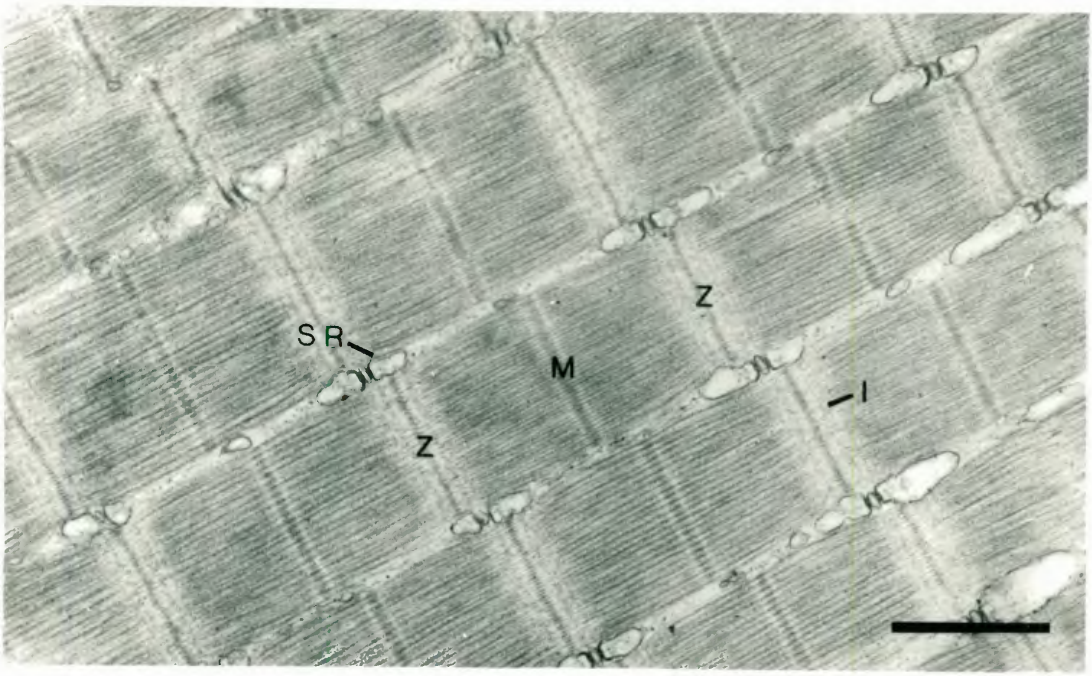
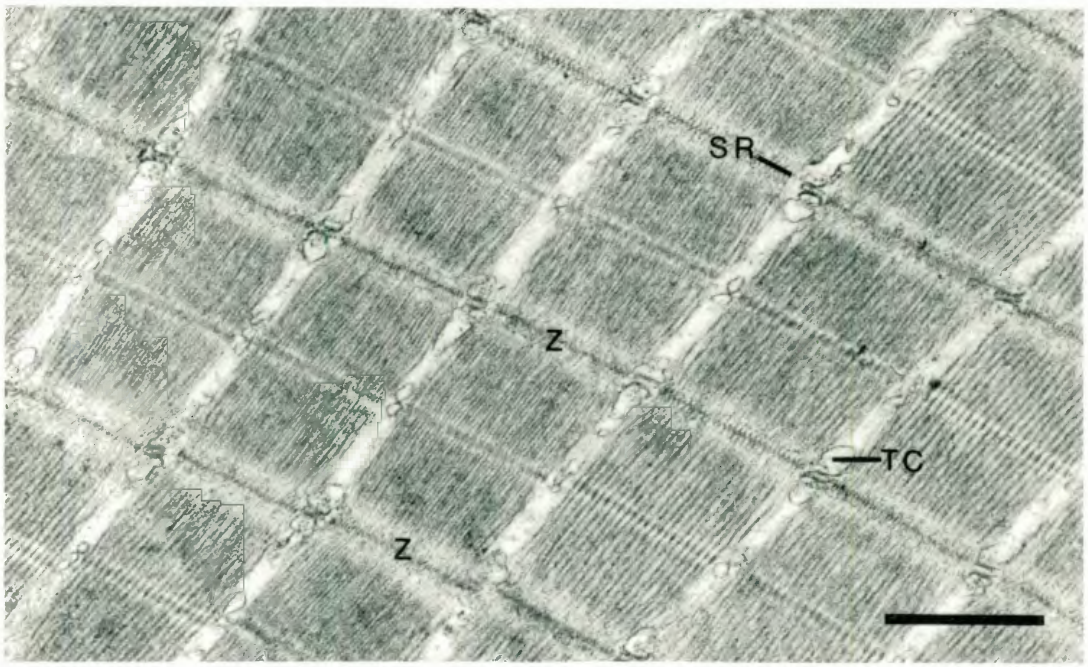

Mean ventilation rate/min
250
225
200
175
150
125
100
75
50
25
0
0
20
40
60
80
100
120
Time in minutes
control set
saline set
high dose

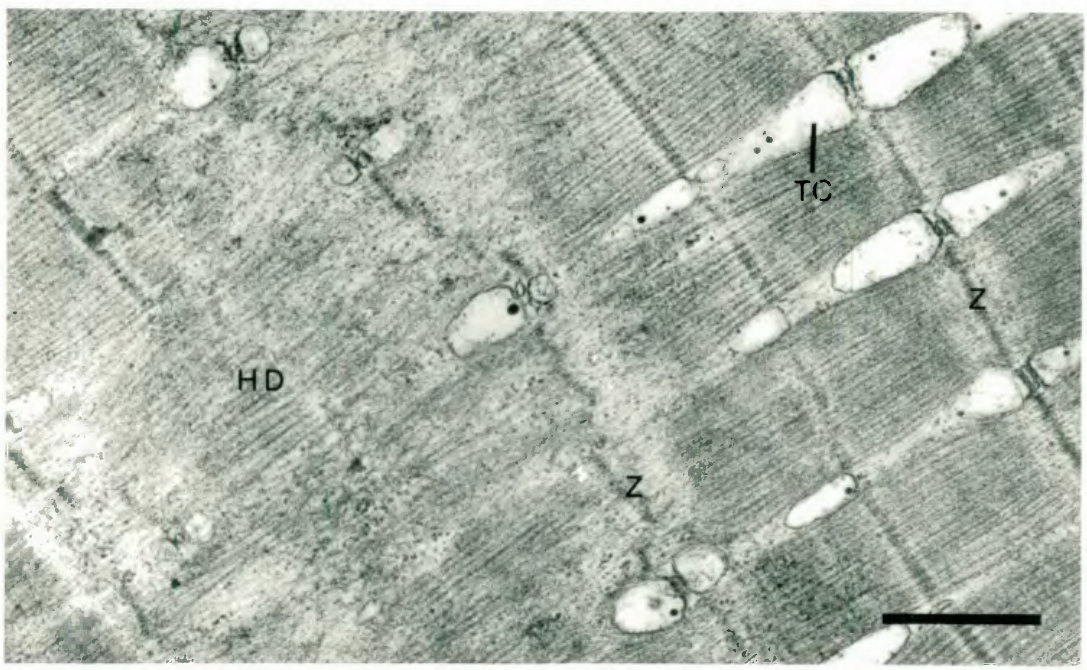
Figure 3.2: Control group for *Carassius auratus* showing normal sternohyoideus muscle tissue, I – I band, M – M line, SR – sarcoplasmic reticulum, Z – Z line (Bar = 0.65 μm).

Figure 3.3: Saline-injected group for *Carassius auratus* showing normal sternohyoideus muscle tissue, SR – sarcoplasmic reticulum, TC – terminal cisternae, Z – Z line (Bar = 0.65 μm).

Figure 3.4: Envenomated group for *Carassius auratus* showing damages of sternohyoideus muscle tissue as the result of myotoxic components, HD – hyaline degeneration, TC – terminal cisternae, Z – Z line (Bar = 0.5 μm).


SR
Z
M
Z
I


SR
Z
TC
Z


TC
Z
HD
Z

Chapter 4

Comparative Studies With Marine Prey Species

4.1 Introduction

Comparative testing was employed to examine the effects of different dosages of *Aipysurus laevis* venom on selected species of its prey.

The overall purpose of this part of the study was:

1. to develop techniques for the collecting, maintaining and handling of the marine species.
2. to compare lethal dose levels among natural prey species, through LD50 values.
3. to compare time to death for different dosages of venom among natural prey species.
4. to obtain information on dose ranges effective for each marine species for use in planning future experiments.
5. to determine which species was most suitable for future investigations.
6. to define behavioural criteria for assessment of degree of envenomation.

4.2 Materials and Methods

A series of progressive dosages of venom was administered to experimental animals over a 48 hour period, and percent mortalities and time to death determined. LD50 values were calculated determining the amount of venom required to kill 50% of the experimental animals over a 48 hour period (Sutherland, 1983). Forty-eight hours is sufficient time, according to Christensen (1968), for the lethal action of the venom to diminish.

The five prey species of fish used in the study were *Chromis nitida* (Fig. 2.6), *Chromis atripectoralis* (Fig. 2.7), *Dascyllus aruanus* (Fig. 2.8), *Istiblennius meleagris* (Fig. 2.9) and *Istiblennius edentulus* (Fig. 2.10). They were obtained and maintained according to procedures described in sections 2.3 and 2.4 respectively.

Twelve fish of each species were injected as outlined in section 2.2, and were immediately transferred to experimental holding facilities (Section 2.5). The fish were observed for 48 hours and detailed behavioural observations recorded for future studies, along with time of occurrence of any deaths.

After each experimental run a progressively lower venom dose was administered to twelve new fish of the same species. This progression of lower doses was continued until a dose was reached that produced no fish mortalities. A further requirement was that at least four of the doses produced between 16 and 84 percent survivorship over the 48 hour period.

Regular controls and saline-injected controls were also observed for 48 hours to determine if handling, injections or the experimental holding facility contributed to the experimental outcome.

Probit analysis, as described by Finney (1971), was used for determining LD50 values for the prey species. A test of heterogeneity was used to determine if the probit lines fitted. Assessment of parallelism was carried out to determine if the lines possessed the same slopes, and for those that did a test of position was then performed. The Maximum Likelihood Program (M.L.P.), developed by Ross (1980), was used for all calculations. The M.L.P., using a logistic curve as a model, was also used for comparing the lines of mean time to death with increasing venom doses for

the five prey species.

4.3 Results

Controls demonstrated no observable problems or mortalities resulting from handling and injection (Section 2.2) or from the experimental holding facilities (Section 2.5), providing procedures for whitespot prevention (Section 2.6) were adhered to.

The test for heterogeneity (Table 4.1) indicates a successful fit of all probit lines used for the computations of LD50 values. All non-significant results are reported as $P > 0.05$.

Figure 4.1 shows the probit curves for the five prey species. *C. nitida*, *D. aruanus* and *I. endentulus* are the most resistant to the venom, showing more gradually sloping curves (Fig. 4.1 and Table 4.1) and higher LD50s (Table 4.1) and fiducial limits (Fig. 4.2) than did *C. atripectoralis* and *I. meleagris*. *D. aruanus* proved to be the most resistant, and *C. atripectoralis* the least. *I. edentulus* was the second most resistant, with *C. nitida* and *I. meleagris* following in decending order. The skewedness of the F.D. limits (Fig. 4.2) is the result of the back-transformation to the original scale from the log transformations required for the computations. Note that the F.D. limits are symmetrical about the LD50 value in the log scale, but when the means were back-transformed to the original scale the limits become skewed. Appendix A presents all of the LD values for the five species.

Since the tests of fit (Table 4.2) were non-significant comparisons of probit lines were able to be made from the models.

There is no significant difference between the slopes of the probit lines (Table 4.2), for *C. nitida*, *D. aruanus* and *I. meleagris*, with *C. nitida* and *D. aruanus* shown to be most closely related. Neither *C. nitida* nor *D. aruanus* are parallel with *C. atripectoralis* or *I. edentulus*, however. *I. meleagris*, *I. edentulus* and *C. atripectoralis* proved to be parallel ($P > 0.05$) with *I. edentulus* and *C. atripectoralis* being most closely related.

Only *C. atripectoralis* and *I. meleagris* show no significant difference with respect to intercepts. This relationship can also be seen with probit line intercepts

in figure 4.1. All other probit lines have significantly different intercepts at the 0.05 level. *D. aruanus* and *I. endentulus* do not share similar intercepts, despite the significant value seen in Table 4.2, since they are not parallel and do not share similar slopes.

Comparisons of LD50 values (Table 4.3) for the parallel probit lines show significant differences at the 0.05 level between *C. nitida* and *D. aruanus*, and at the 0.01 level for *C. nitida* and *I. meleagris*, *D. aruanus* and *I. meleagris*, *C. atripectoralis* and *I. edentulus* and for *I. edentulus* and *I. meleagris*. Only *C. atripectoralis* and *I. meleagris* exhibited no significant difference between LD50 values, which reflects the similarities between the two species in their reaction to the venom. *D. aruanus* and *C. nitida* are also similiar in response, with a significant difference at the 0.05 level only. All other comparisons show a very high significant difference ($P < 0.01$), meaning venom response is different. *C. atripectoralis* and *I. edentulus* demonstrate the greatest difference, followed by the comparisons of the two *Istiblennius* species.

Further comparisons of the prey species were made by examining the mean time to death with increase in venom dosage (Fig. 4.3). At the highest dose, all species were quickly affected, with the two *Istiblennius* species reacting the slowest, and the two *Chromis* species the quickest. At the lower doses *I. meleagris* demonstrated a relative change in position and reacted the quickest to the venom.

Of the three pomacentrids, *D. aruanus* proved to be the most resistant, being the slowest to react to the venom at all doses, whereas *C. atripectoralis* was the least resistant.

There was no significant difference in the slopes of the lines in figure 4.3, with no significant difference between *C. nitida*, *C. atripectoralis* and *D. aruanus* at the 0.05 level (Table 4.4). *I. meleagris* and *I. edentulus* also showed no significant difference, and are also parallel to both *C. nitida* and *D. aruanus* ($P > 0.05$). *C. atripectoralis* possesses a significantly different slope than that of the *Istiblennius* species ($P < 0.05$).

No mortality occurred in any of the control subjects during the experiment and none showed any behavioural signs of stress.

4.4 Discussion

The study provided valuable information on comparisons of the effects of *A. laevis* venom on the prey species, as well as an excellent base of information and experience required for further studies. It also proved valuable for formulating ideas and directions for future behavioural examinations of envenomated prey.

The range of resistance to the venom, as seen by the probit curves (Fig. 4.1), fiducial limits (Fig. 4.2) and LD50 values (Table 4.1) may be in part due to the different habitats and life styles of the fish. Although no quantative data were collected, observations during collecting, handling and retention in holding facilities demonstrated *D. aruanus* as being the hardiest and *C. atripectoralis* as the least hardy of the prey species. *D. aruanus* is also the most resistant, as shown by the study, with lower oxygen requirements than *C. atripectoralis*, which possess more erratic behaviour, with a tendency to hide and is the least resistant to the venom. *C. nitida*, a hardy species, is also more resistant than *C. atripectoralis*. Of the two *Istiblennius* species, *I. edentulus*, the most resistant, was found under rocks and coral furtherest removed from the water's edge reflecting its ability to withstand harsher conditions. From aquarium observations *I. edentulus* proved to be the hardiest of the two.

The parallel relationships between *C. nitida*, *D. aruanus* and *I. meleagris* suggest similar profiles, with respect to percent mortalities and increasing venom dose. The close relationship between *C. nitida* and *D. aruanus* can also be seen on the graph of probit curves (Fig. 4.1) and in the LD50 comparisons in table 4.3. No parallel relationship was seen when *C. nitida* and *D. aruanus* were compared to *C. atripectoralis* and *I. edentulus*, implying different profiles of venom effects on percent mortalities. *I. meleagris* did show similarities of slope and thus similarities in profiles of venom effects, with *C. atripectoralis* and *I. edentulus*. In addition it shared points with *C. atripectoralis* (Table 4.2) (Figs. 4.1 and 4.2), with no significant difference between the LD50 values of the two species (Table 4.3).

The profile similarities for mean time to death and increasing venom dose for *C. nitida* and *C. atripectoralis* (Fig. 4.3) and through line comparisons (Table 4.4) indicates similar reactions to the venom. *C. nitida* and *D. aruanus* also demonstrate a

parallel relationship with the two *Istiblennius* species, which themselves share similar profiles, as seen in Table 4.4. Figure 4.3 shows that the closest affinities between the two groups occurs in the middle to low dose range, with the greatest differences occurring in the high.

C. atripectoralis is significantly different from the two *Istiblennius* species, not sharing the same profiles of mean time to death with increasing venom doses. The significant difference in displacement (Table 4.4) indicates that the differences in slopes are not the result of their distance apart.

All species were quickly affected by venom at the higher doses. The *Istiblennius* species demonstrated the most resistance (Fig. 4.3) partly due to the possession of cutaneous respiration (Graham, 1976), allowing for longer survival time after gill ventilation ceased, and to the lower oxygen requirements dictated by their secretive life style (Fry, 1957; Moyle and Cech, 1982). Once gill ventilation ceased, however, they survived only a while longer then the others, and death was inevitable. Cutaneous respiration is not efficient enough on its own, according to Moyle and Cech (1982), to maintain sufficient oxygen levels for an indefinite time while in the water, even though it allows the *Istiblennius* species to successfully exist out of the water (Norman, 1975). The reasons, according to Norman (1975), is that air is much more suitable than water for respiration, having approximately 25 times more oxygen / volume, with less energy required to obtain it.

Of the three pomacentrids, *D. aruanus* demonstrated the most resistance in the high and middle doses (Fig. 4.3) with *C. atripectoralis* showing the least. This parallels the previous results of the LD50 studies, as well as the fish's life styles, with *D. aruanus* again being the hardiest of the group.

In the middle and the low doses the species profiles are more similar, with the exception of *I. meleagris*, which shows a quicker reaction to the venom. The pomacentrids are more resistant than the *Istiblennius* species, which show a reversal from the higher doses. Possibly at these doses other venom components other than neurotoxins are more important, with a lessening of importance of the neurotoxic elements. At the higher doses the neurotoxin's effects are very quick with death resulting from the shutdown of the gill ventilation system. When doses are low enough that animals

survive the neurotoxin other toxins may be allowed to exert their effect.

D. aruanus, *C.nitida* and *I. meleagris* were chosen for further studies of changes in ventilation rates resulting from envenomation. The choice was based on their resistance to the venom, ease of capture and handling, ease of observation and their parallel relationships. Also, slope similiarities and greater availability led to the choosing of *I. meleagris* over *I. edentulus* (the more resistant of the two).

Because of *D. aruanus'* higher resistance to the venom and its robustness, it was used in more advanced studies of electron microscopy and venom fractions.

C. atripectoralis was the least suitable for any further investigation, demonstrating the lowest resistance to the venom as well as being the most difficult to collect, maintain and observe.

Figure 4.1: All species' probit curve determined by probit analysis using the Maximum Likelihood Program.

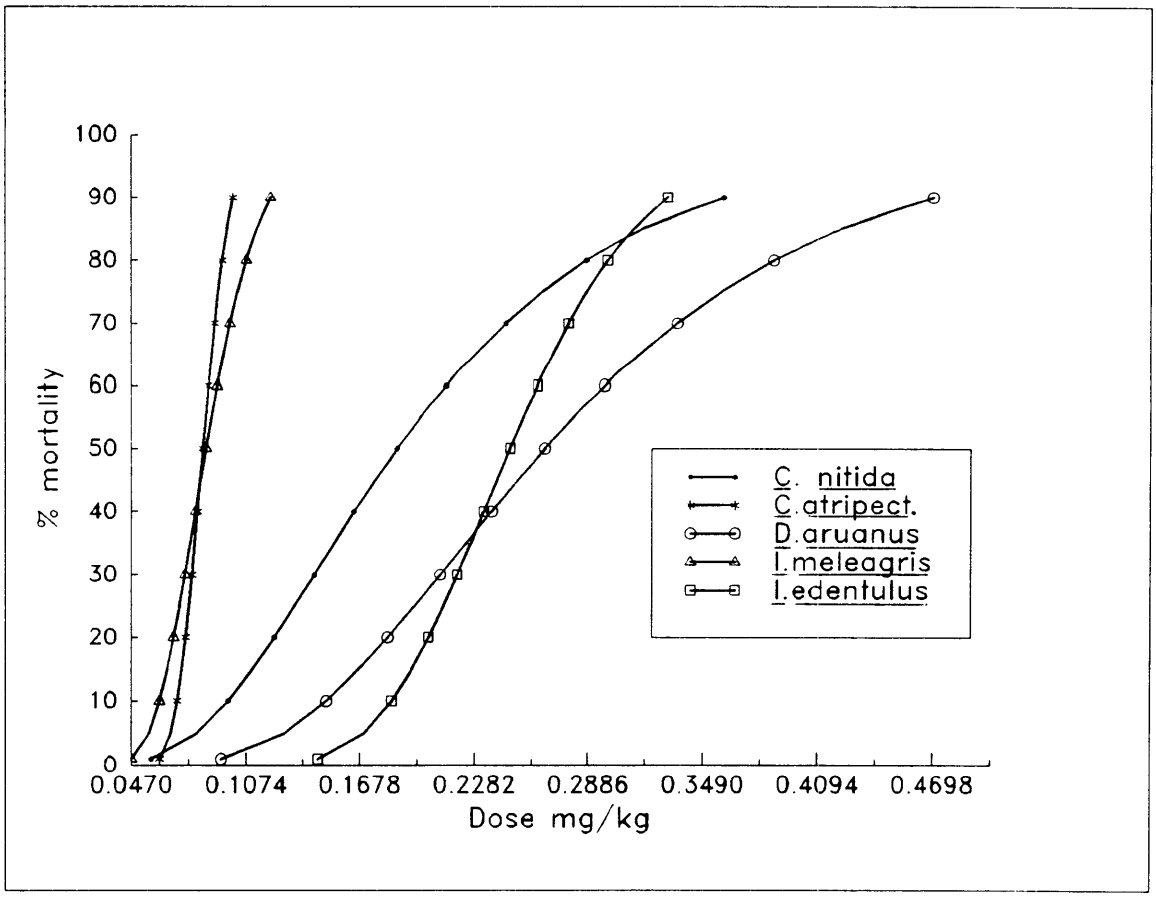

100
90
80
70
60
50
40
30
20
10
0
% mortality
0.0470
0.1074
0.1678
0.2282
0.2886
0.3490
0.4094
0.4698
Dose mg/kg
C. nitida
C.atripect.
D.aruanus
I.meleagris
I.edentulus

Figure 4.2: Fiducial limits determined for the LD50 values for all five prey species from probit analysis using the Mamimum Likelihood Program.

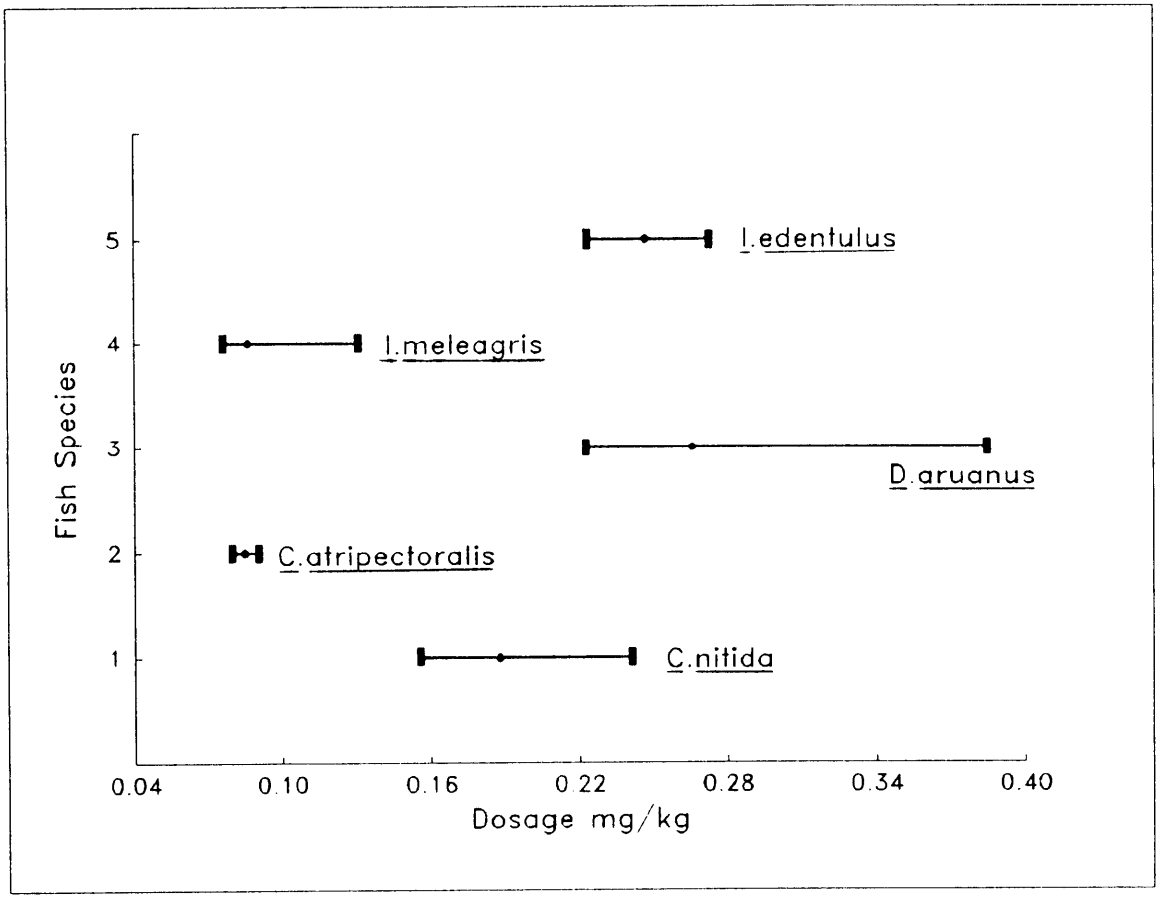

Fish Species
5
4
3
2
1
I.edentulus
I.meleagris
D.aruanus
C.atripectoralis
C.nitida
0.04
0.10
0.16
0.22
0.28
0.34
0.40
Dosage mg/kg

Table 4.1: Probit analysis results for *Chromis nitida*, *Chromis atripectoralis*, *Dascyllus aruanus*, *Istiblennius meleagris* and *Istiblennius edentulus*, showing test of heterogeneity for model fit, line slope, LD50 values and fiducial limits. (F.D. – fiducial limits, DF – degrees of freedom, X^2 – chi-square)

Species	Heterogeneity X^2	DF	Slope	LD50	F.D. Limits
C. nitida	4.189	4	-11.84793	0.18746	0.2411–0.1560
C. atripectoralis	1.949	5	-91.66439	0.08455	0.0904–0.0800
D. aruanus	1.472	5	-7.84370	0.26559	0.3842–0.2232
I. meleagris	2.629	4	-47.55696	0.08615	0.1312–0.0764
I. edentulus	1.352	4	-19.16953	0.24693	0.2721–0.2235

Figure 4.3: Comparisons of mean time to death with different venom doses for the five prey species.

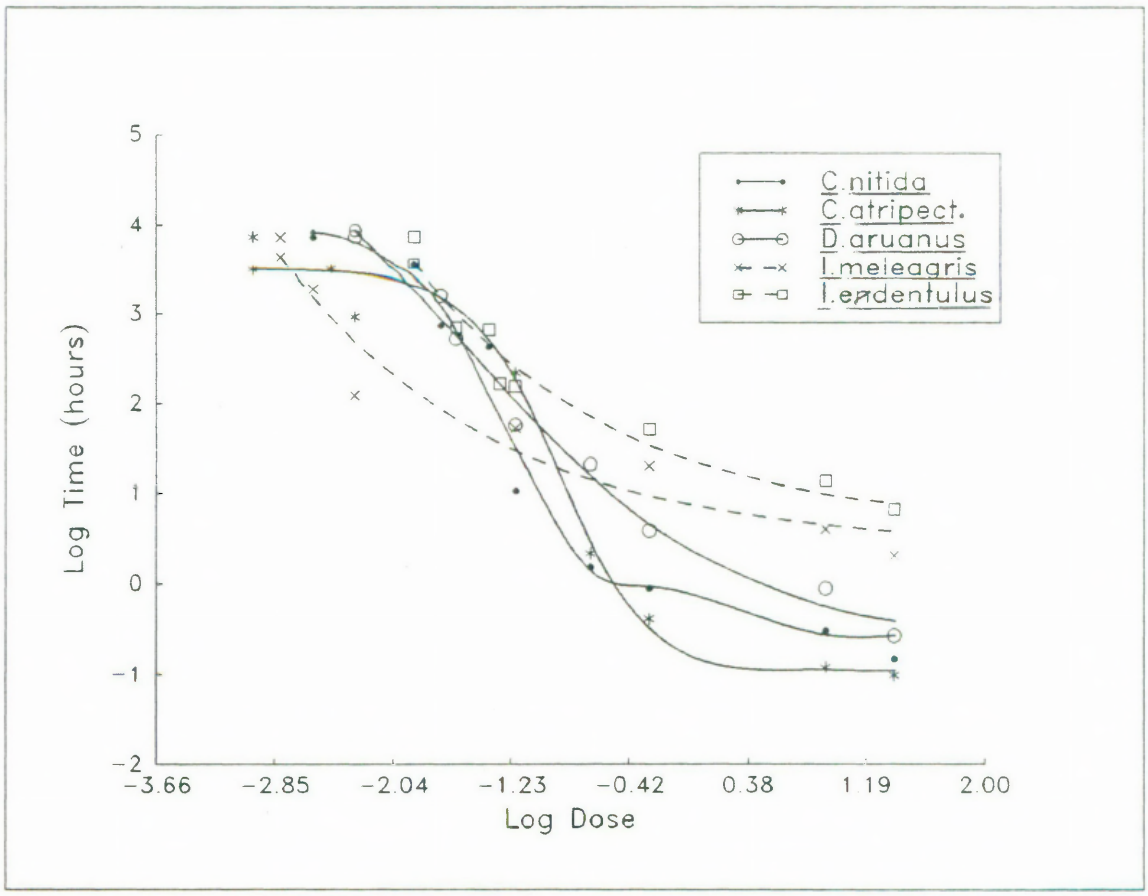

C.nitida
C.atripect.
D.aruanus
I.meleagris
I.edentulus
Log Time (hours)
5
4
3
2
1
0
−1
−2
−3.66
−2.85
−2.04
−1.23
−0.42
0.38
1.19
2.00
Log Dose

Table 4.2: Probit line comparisons for *Chromis nitida*, *Chromis atripectoralis* , *Dascyllus aruanus*, *Istiblennius meleagris* and *Istiblennius edentulus* showing test of heterogeneity for model fit, test of parallelism and test of position. (DF – degrees of freedom, X^2 – chi-square)

Species Comparisons	Heterogeneity X^2	DF	Parallelsim X^2	DF	Position X^2	DF
C. nitida *C. atripectoralis* *D. aruanus* *I. meleagris* *I. edentulus* Analysis of x^2	11.59	22	14.81	4**	69.02	4**
C. nitida *D. aruanus* *I. meleagris* *I. edentulus* Analysis of x^2	9.64	17	6.09	3	40.18	3**
C. nitida *C. atripectoralis* Analysis of x^2	6.14	9	10.48	1**	20.89	1**
C. nitida *D. aruanus* Analysis of x^2	5.66	9	0.16	1	7.95	1**
C. nitida *I. meleagris* Analysis of x^2	6.82	8	1.94	1	11.91	1**
C. nitida *I. edentulus* Analysis of x^2	5.54	8	4.74	1*	7.07	1**
C. atripectoralis *D. aruanus* Analysis of x^2	3.42	10	9.00	1**	38.25	1**
C. atripectoralis *I. meleagris* Analysis of x^2	4.58	9	2.56	1	0.52	1
C. atripectoralis *I. edentulus* Analysis of x^2	3.30	9	1.95	41	44.09	1**
D. aruanus *I. meleagris* Analysis of x^2	4.10	9	1.35	1	26.63	1**
D. aruanus *I. edentulus* Analysis of x^2	2.82	9	3.50	1	0.08	1
I. meleagris *I. edentulus* Analysis of x^2	3.98	8	0.11	1	29.34	1**

* ($P < 0.05$)
** ($P < 0.01$)

Table 4.3: Comparisons of LD50 values determined by probit analysis for *Chromis nitida*, *Chromis atripectoralis*, *Dascyllus aruanus*, *Istiblennius meleagris* and *Istiblennius edentulus*. (S.E. – standard error)

Comparison	LD50(log)	S.E.(log)	Z Values
C. nitida	−0.72708	0.04187	2.49*
D. aruanus	−0.57580	0.04310	
C. nitida	−0.72708	0.04187	5.97**
I. meleagris	−1.06475	0.03693	
D. aruanus	−0.57580	0.04310	8.50**
I. meleagris	−1.06475	0.03693	
C. atripectoralis	−1.07291	0.01071	0.21
I. meleagris	−1.06475	0.03930	
C. atripectoralis	−1.07291	0.01071	20.80**
I. edentulus	−0.60742	0.01882	
I. meleagris	−1.06475	0.03930	10.79**
I. edentulus	−0.60742	0.01882	

* Statistically significant ($P < 0.05$)
** Statistically significant ($P < 0.01$)

Table 4.4: Mean time to death with venom dose comparisons for *Chromis nitida*, *Chromis atripectoralis*, *Dascyllus aruanus*, *Istiblennius meleagris* and *Istiblennius edentulus*, showing common nonlinear relationship and displacement values. (DF – degrees of freedom)

Species Comparison	Common Nonlinear F Value	DF	Displacement F Value	DF
C. nitida *C. atripectoralis* Values:	2.72	2,9	0.12	2,9
C. nitida *D. aruanus* Values:	2.19	2,9	3.82	2,9
C. nitida *I. meleagris* Values:	4.19	2,8	10.69**	2,8
C. nitida *I. edentulus* Values:	2.23	2,9	25.68**	2,9
C. atripectoralis *D. aruanus* Values:	3.15	2,8	6.77*	2,8
C. atripectoralis *I. meleagris* Values:	5.34*	2,7	12.21**	2,7
C. atripectoralis *I. edentulus* Values:	5.72*	2,8	38.15**	2,8
D. aruanus *I. meleagris* Values:	0.28	2,7	0.43	2,7
D. aruanus *I. edentulus* Values:	0.13	2,8	0.25	2,8
I. meleagris *I. edentulus* Values:	1.41	2,7	11.23**	2,7
C. nitida *C. atripectoralis* *D. aruanus* Values:	2.39	4,13	3.17	4,13
C. nitida *C. atripectoralis* *D. aruanus* *I. meleagris* Values:	4.38**	6,16	6.1**	6,16
C. nitida *C. atripectoralis* *D. aruanus* *I. edentulus* Values:	2.78**	6,16	14.08**	6,16

* Statistically significant ($P < 0.05$)
** Statistically significant ($P < 0.01$)

Chapter 5

Ventilation Studies

5.1 Introduction

One of the more obvious effects of sea snake envenomation is the shutdown of the ventilation system (Section 1.1). In light of this, it was decided that a closer examination of this mechanism be carried out.

Preliminary studies using goldfish (Chapter 3) demonstrated definite changes in the ventilation rates of envenomated fish (Fig. 3.1). The present chapter reports on comparative studies on envenomated marine prey species of *Aipysurus laevis*.

5.2 Materials and Methods

The three marine prey species used were the same as for the comparative study (Chapter 4), i.e., *Chromis nitida* (Fig 2.6), *Dascyllus aruanus* (Fig. 2.8) and *Istiblennius meleagris* (Fig. 2.9). The fish were obtained and maintained according to procedures described in sections 2.3 and 2.4 respectively.

Five experimental sets were employed for each species, with twelve fish per set, using a control, saline-injected control, and a low, medium and high dose of venom. The doses of *A. laevis* venom used (Figs. 5.1, 5.3 and 5.5) were determined from the LD50 calculations of chapter 4; a complete listing of LD values is shown in Appendix A. The fish were injected according to procedures outlined in section 2.2

and immediately transferred to experimental holding facilities (Section 2.5).

Movements of the mouth and/or opercular shields, which are synchronized and nearly simultaneous (Hughes and Shelton, 1958), were recorded as ventilation rates per minute, using a Micronta Electronic L.C.D. digital countdown timer.

Observations of each experimental set were carried out for 48 hours in accordance with the LD50 studies of chapter 4; Christensen (1968) indicates this as a sufficient time course for the lethal action of venom.

The time periods for the experimental sets were determined from previous observations from the comparative studies (Chapter 4), with shorter time periods being used in the beginning so that initial venom effects were not overlooked. The actual time periods can be seen in Tables 5.1 and 5.2.

The order in which fish were observed for each time period was selected randomly using computer-generated random numbers for the 12 subjects. Except for the beginning time periods the fish were allowed to become accustomed to the observer's presence for 15 to 20 minutes before observations took place. The experimental areas were sectioned off in order to prevent outside interference.

Ventilation rates of each species were graphed, comparing the five experimental sets. One set of graphs used means for the total number of fish per experimental set, and the second used means only for the fish still surviving at the time of observation. Other graphic representations compared the variability of all fish within each experimental set.

Analysis of repeated measurements, as outlined by Jennrich *et al.* (1983), was used to fit the species' experimental profile to a polynomial model to detect differences between experimental groups over time. It produces the necessary test power required, according to Allen *et al.* (1983) and Gill (1986), when subject number is low, as in this study. An L.S.D. program, using one-way analysis of variance, determined where actual significant differences were located. From this, comparisons between experimental groups were carried out.

5.3 Results

5.3.1 Mean ventilation rates: total number of fish

Figures 5.1 through 5.6 show mean ventilation rates for *C. nitida*, *D. aruanus* and *I. meleagris* determined from the total number of fish in each experimental set. These results represent the responses of the dying fish to envenomation, since the mortalities (ventilation rate zero) affected the mean ventilation rates. Figures 5.1, 5.3 and 5.5 show the profiles for the entire 48 hours (2880 minutes), with figures 5.2, 5.4 and 5.6 showing the first 400 to 450 minutes at a larger scale for easier interpretation of the beginning observations.

The controls, saline-injected controls and fish with low venom doses had similar profiles, with high initial ventilation rates, due to subject manipulation, but with an eventual leveling off. The low venom dose resulted in higher ventilation rates than in the controls, especially near the end of the study. The medium doses led to an increase in ventilation rates at first, followed by a decrease that eventually dropped below those of the controls. *D. aruanus* (Fig. 5.3) and *I. meleagris* (Fig. 5.5) showed the greatest differences from the controls.

The high dose groups had very high ventilation rates, followed by a steady decrease and an eventual levelling off at or near zero. *I. meleagris* (Fig. 5.6) showed the quickest reaction, reaching zero at approximately one hour. *C. nitida* (Fig. 5.2) reached zero in four hours. *D. aruanus* (Fig. 5.4) showed the slowest reaction, never actually reaching zero due to the survival of one fish.

Table 5.1 shows the mean ventilation rates (determined from all the fish in the experimental set) and the respective standard deviations, along with the time intervals used for the study. Appendix B presents graphic profiles of all the fish ventilations, showing variability between the individuals of the set. The control, saline-injected and low dose groups exhibited lower variability than did the medium and high dose ones. A marked increase in variability is observed with an increase in dosage and time.

5.3.2 Mean ventilation rates: surviving fish only

Figures 5.7 through 5.12 show graphic profiles of mean ventilation rates for *C. nitida*, *D. aruanus* and *I. meleagris* respectively, with the means determined only from the fish still living at a particular observation time, i.e., zeros from dead fish are excluded. Figures 5.7, 5.9 and 5.11 show the profiles for the entire 48 hours (2880 minutes); figures 5.8, 5.10 and 5.12 show the first 400 to 450 minutes at a larger scale, allowing for easier interpretation of the beginning observations.

The results from these studies can be divided into three divisions according to ventilation changes (Fig. 5.13) as follows:

Division 1 (Initial effects): High ventilation rates probably as the result of subject manipulation, lack of familiarization to the experimental conditions and presence of observer. The rates eventually decreased. Controls, saline-injected controls and fish with low venom doses best demonstrated this.

Division 2 (Primary venom effects): High ventilation rates as the result of the major components of the venom, i.e., neurotoxins. Fish with medium and high venom doses best demonstrated this.

Division 3 (Residual venom effects): Long term venom effects with higher than normal ventilation rates resulting from initial damage and/or minor venom components. Fish with medium and low venom doses best demonstrated this. Also seen in survivors of high doses.

No mortality occurred among the control, saline-injected and low venom groups (Figs. 5.7, 5.9 and 5.11) and the results were similar to those of section 5.3.1. High ventilation rates were observed in division 1 probably as the result of the initial handling and injection procedures, with an eventual leveling off.

Both the medium and high doses produced different profiles than in section 5.3.1, especially in division 2 and 3, where there was considerable mortality.

The medium dose caused an initial increase in ventilation rates, as in section 5.3.1, but with a longer duration. This range of sustained high ventilation rates defined the boundries of division 2 in the model in figure 5.13, and are possibly

primary venom effects. The rates eventually decreased and leveled off, defining division 3, but remained higher than for the controls, saline-injected and low venom groups. This division is thought to be the result of the residual effects of the venom. In division 3 *C. nitida* (Fig. 5.7) and *I. meleagris* (Fig. 5.11) showed the largest differences between fish in medium dose groups, and those in control and low dose groups.

High venom dose resulted in high ventilation rates (division 2), and with the exception of *I .meleagris*, were maintained longer than in section 5.3.1. Both *I. meleagris* and *C. nitida* ventilation rates dropped to zero when all fish died, but *D. aruanus* maintained a higher ventilation rate in division 3, similar to the medium dose profile, because of one surviving fish.

Table 5.2 shows the mean ventilation rates of surviving fish, along with the standard deviations and 'n' values for the medium and high dose groups for each species. Data from the control, saline-injected and low dose groups can be seen in table 5.1.

5.3.3 Statistical analysis

Repeated measures (Jennrich *et al.,* 1983) was carried out as a split plot over time. However, since a test of sphericity proved highly significant ($P< 0.01$) it was necessary to use a Greenhouse Geisser correction. The analysis of ventilation rates indicates a highly significant difference in treatment over time ($P< 0.01$).

Due to the nonhomogeneity of variance, indicating the dependence of the standard deviations on the means for *D. aruanus* and *C. nitida*, transformations were used (Allen *et al.,* 1983), with inverse transformations proving to be the most appropriate. No transformations were required for *I. meleagris*.

Probability values obtained with one-way analysis of variance were used for comparisons between experimental groups (Appendix C). The values were then presented graphically in Appendix D, and the percent of the total time that the group comparisons showed no significant differences ($P> 0.05$) is seen in table 5.3.

For obtaining the following comparisons between experimental groups, Appendix D, table 5.3 and figures 5.7 to 5.12 were employed, with all significance at the 0.05

level. The total experimental time (T.E.T.) was 48 hours (2880 minutes), and an example of the three divisions of ventilation change is seen on figure 5.13. High ventilation rates in division 1 are probably due to subject manipulation and habitat familiarization, in division 2 to the primary effects of the venom and in division 3 to residual venom effects.

5.3.4 *Dascyllus aruanus*

Comparisons between the experimental groups of *D. aruanus* (Table 5.3, Fig. 5.9 and Appendix D) showed the control and saline-injected groups to have no significant difference 90% of the T.E.T.. The significant differences that were present occurred in divisions 1 and 3. The control and low dose (0.01 mg/kg) groups demonstrated no significant difference 63% of the T.E.T., with 90% of the differences occurring in division 3, as the result of an increase in ventilation at the low dose (Fig. 5.9). The remainder of the differences occurred in divisions 1 and 2. The control and medium dose (0.3 mg / kg) groups showed no significant difference 8% of the T.E.T.. The similarities are found in division 1, with all of division 2 and the majority of division 3 demonstrating significant differences. The control and the high dose (0.75 mg / kg) groups demonstrated no significant difference 33% of the T.E.T., with division 3 showing 100% of the similarities as the result of the one surviving fish eventually achieving a rate near normal (Fig. 5.9). There are no similarities if the sole survivor is excluded, with 100% mortality at 1200 minutes.

The saline-injected and low dose comparison of *D. aruanus* showed no significant difference 83% of the T.E.T., with approximately 80% of the differences occurring in division 3. As with the comparison of the control and low dose groups, this difference is probably the result of the increase in the ventilation rate of animals receiving a low dose. The saline-injected and low dose groups are more similar than are the control and the low dose ones. This similarity is possibly due to both the saline-injected group and low dose group showing handling effects. The saline-injected and medium dose groups demonstrated no significant difference 48% of the T.E.T. All of division 2 and 22% of division 3 showed a significant difference, with the ventilation rates of fish of the medium dose slightly higher than those that

were saline-injected. Comparison with the high dose group showed no significant difference 46% of the T.E.T., which, as in the case of the control and the high dose groups is attributable to the surviving fish. Otherwise, all fish died at 1200 minutes and no similarities occurred between the two groups.

The low dose group compared to the medium and high dose groups showed no significant difference 41% and 43% of the T.E.T. respectively. Almost all of the differences occurred in division 2, with similarities occurring with the high dose group in division 3, again attributable to the one surviving fish of the high dose group.

The medium and high dose groups showed no significant difference 93% of the T.E.T., their profiles being similar because of the one suvivor of the high dose. The highest ventilations occurred in division 2, with both groups remaining high in division 3 (Fig. 5.9).

5.3.5 *Istiblennius meleagris*

Comparisons of *I. meleagris* (Table 5.3, Fig. 5.11 and Appendix D) showed no significant difference 97% of the T.E.T. between the control and saline-injected groups, with only minor differences occurring. The control and the low dose (0.01 mg / kg) groups showed no significant difference 60% of the T.E.T., with 88% of the differences occurring in division 2 and the remainder in division 3. Comparison of the controls with the medium dose (0.1 mg / kg) and the high dose (0.75 mg / kg) groups demonstrated significant differences 100% of the T.E.T..

The saline-injected and low dose groups showed no significant difference 84% of the T.E.T., with 91% of the differences occurring in division 2, and the remainder occurring in division 3. The saline and the low dose groups are more similar in their profiles than are the control and low dose groups. This similarity is possibly due to both the saline-injected and the low dose groups showing handling effects. Ventilation rates of the low dose group were higher than those of the control and the saline-injected groups the majority of the time (Fig. 5.11). Comparisons of the saline-injected group with the medium and high doses groups demonstrated significant differences 99% and 100% of the T.E.T. respectively. The low dose group

is significantly different from the medium and high dose groups 100% of the T.E.T. The medium and the high dose groups also were always significantly different from each other.

When considering the 120 minute survival time of the high dose group, the significant difference with the medium dose group drops to 87%, as it did with the saline-injected group. This drop is the result of the lines of ventilation rates crossing over (Fig. 5.12) during the decrease in the rates of the high dose group, producing the lower significant differences due to the proximinities of the rates. This does not reflect actual similarities between the groups, since the high dose fish were dying whereas the other two groups of fish were not.

Ventilation rates of fish with a medium dose were very high during division 2. They subsequently decreased in division 3, although remaining higher than in other groups. The high dose group had an extremely high mean ventilation rate, which dropped to zero within 120 minutes.

5.3.6 *Chromis nitida*

Comparisons of *C. nitida* (Table 5.3, Fig. 5.7 and Appendix D) showed the control and saline-injected groups to have no significant difference 45% of the T.E.T. This low percentage may be in part due to the closeness of the control and low dose (0.01 mg / kg) groups, which showed no significant difference 93% of the T.E.T., making the saline group appear further away than it actually was. Figure 5.7 shows the close proximities of the ventilation rates of the three experimental groups, with an increase in ventilation of the saline-injected group in division 3 where the highest percent of significant differences occurred. The control and low dose groups demonstrated their differences in division 1 and early parts of division 2, with no differences in the remainder of the T.E.T. The control and the medium dose (0.25 mg / kg) groups were significantly different 98% of the T.E.T., with the only similarity in division 1. The control and the high dose (0.5 mg / kg) groups were significantly different 100% of the T.E.T., but only 94% of the time when considering the actual time of death (240 minutes).

The saline-injected and the low dose groups demonstrated no significant difference 54% of the T.E.T., with 57% of the total differences in division 2 and the remaining 43% in division 3. The saline-injected and low dose groups were more similar than were the control and saline-injected groups. This similarity is possibly due to both the saline-injected and low dose groups showing handling effects. The saline-injected and the medium dose groups demonstrated no significant difference 4% of the T.E.T., with the only similarities occurring for 120 minutes in division 1. The remainder of the T.E.T. they were significantly different. The saline-injected and the low dose groups were both significantly different from the high dose group 100% of the T.E.T. When examining the 240 minute survival time of the high dose, they showed no significant difference 9% and 6% of the time respectively. The similarities are the result of the lines of the ventilation rates (Fig. 5.8) crossing over during the decrease in the rate of the high dose group producing the higher similarities due to the proximinities of the rates. This does not reflect actual similarities between the groups since the high dose fish were dying whereas the other groups were not.

The low and medium dose groups showed no significant difference 4% of the T.E.T., with most of the similarities occurring in division 1, and at the beginning of division 2. In the remainder of division 2 and all of division 3 they proved to be significantly different.

The medium and high dose groups showed significant differences 100% of the T.E.T., with a value of 32% over the 240 minute survival time for the high dose group, which occurred in division 2. Unlike the previous cross-overs, this is not an artificial similarity since both groups demonstrated similarily high ventilation rates in division 2 before the actual decrease of the rates in the high dose group (Fig. 5.8). The medium dose group demonstrated a high ventilation rate throughout division 2, and decreased in division 3, but remained higher than the control, saline-injected and low dose groups.

Appendix E contains the standard error of the means for the three prey species obtained from the one-way analysis of variance.

5.4 Discussion

The high degree of similarity between the control and saline-injected groups rules out the injection medium as contributing to elevated ventilations. The highly significant difference between the control and saline-injected groups on one hand as compared with groups subjected to the medium and high venom dose on the other hand demonstrate the effects of *Aipysurus laevis* venom on the ventilation rates of prey species.

Toxicity of snake venom is considered by Barme (1968), Chang (1979) and Heatwole (1987) to be a synergistic effect of the component parts, with all the components possessing different functions. Some of the components, i.e. neurotoxins, are more powerful than others, and when in high doses over-shadow the effects of the lesser components through quicker action and death resulting from respiratory arrest (Su *et al.*, 1967; Worrel, 1967; Barme, 1968; deVries and Condrea, 1971; Kochva and Gans, 1971; Pickwell, 1972; Datyner and Gage, 1973; Mebs, 1978; Lee and Lee, 1979; Sutherland, 1983). In lower doses the minor effects are able to be expressed, allowing for a better understanding of the other venom constituents. The three divisions of ventilation changes, as described in section 5.3.2, may indicate sequential operation of different venom components.

Venom caused rapid increases in ventilation in division 2, followed by a rapid decrease, with all fish dying from respiratory arrest, except the one surviving *D. aruanus*. These deaths probably were due primarily to the neurotoxic components, which are curare-like in action (Tamiya *et al.*, 1967; Barme, 1968; Lee, 1970; Lee, 1971a; Lee, 1971b; del Castillo and Anderson, 1974; Mebs, 1978; Lee and Lee, 1979), and are the primary agents of muscle paralysis. The other, less important venom components were not expressed before the fish died from the quicker-acting neurotoxins.

The medium doses also resulted in rapid increase in ventilation rates in division 2, but remained high throughout the division. This increase, as in the case of the high dose, was probably the result of the neurotoxin (the primary venom component), but being less potent because of weaker dosages, allowed for a longer survival

time. The ventilation rates eventually decreased, but remained higher than the controls and the saline-injected groups in division 3. Even though the fish survived the primary effects of the venom, complete recovery did not occur, as indicated by the higher ventilation rates in division 3. This profile is thought to be the result of the other slower-acting components of the venom, or the residual effects of the primary venom. It should be noted that not all fish survived the medium dose, suggesting intraspecific differences in resistance to venom.

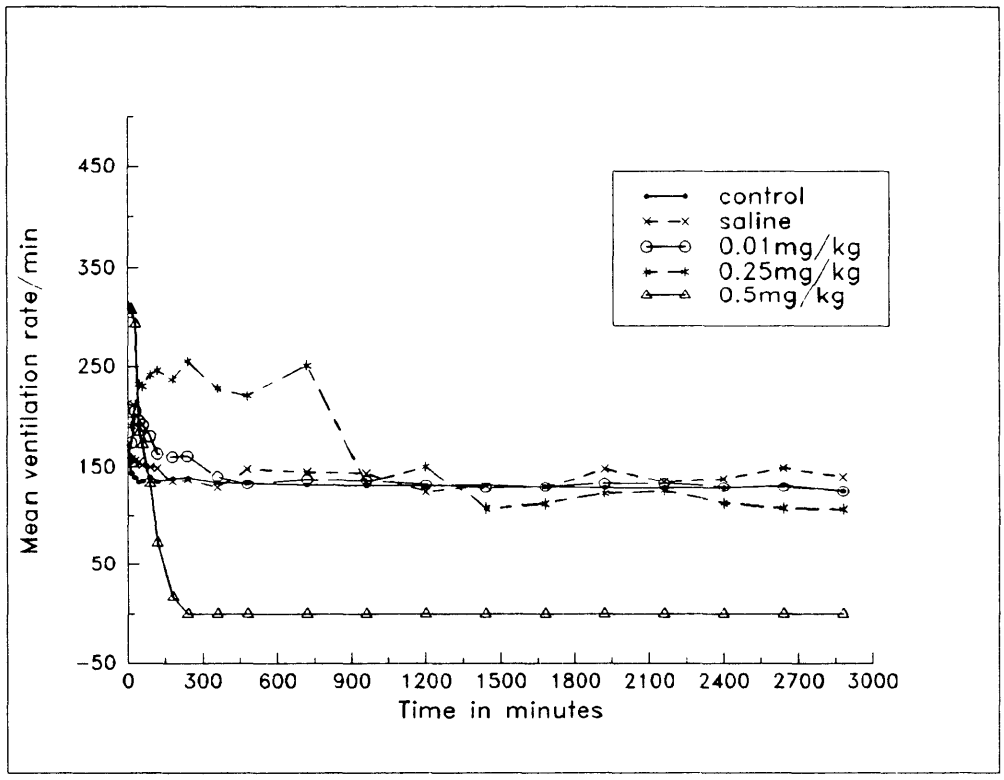
This profile is also seen to a certain degree at low doses. A slight increase in ventilation rates compared to the controls and saline-injected fish, probably resulting from the neurotoxin, can be seen in division 2, with high ventilations in division 3, probably as the result of the other components. The low dose group, however, is statistically more similar to the controls and saline-injected groups then to the medium and high doses groups.

The ventilation profile of one *D. aruanus* (Fig. 5.9) injected with a high venom dose mimicked the medium dose response of the other individuals of that species. This one survivor may have been an unusually resistant individual. This possible resistance to the venom can be seen in other envenomated subjects (see Appendix B, Table 5.1 and 5.2), where an increase in venom dose and time shows an increase in variability in the subjects. These intraspecific differences indicate that the venom is affecting different individuals in different degrees. A few individuals from a population of prey species possess high enough resistance to survive envenomations lethal to others. If predation were high, selection of resistant fish could possibly lead to a resistant population.

Interspecific differences in resistance to venom, as expressed by LD50s and time to death, was observed in chapter 4. *D. aruanus* was shown to be the most resistant to *A. laevis* venom in terms of having greater stability of ventilation rates.

Figure 5.1: Mean ventilation rates / minute for *Chromis nitida* in five different experimental sets over 2880 minutes (n = the total number of fish for each set).

Figure 5.2: Mean ventilation rates / minute for *Chromis nitida* in five different experimental sets for the first 400 minutes (n = the total number of fish for each set).


Mean ventilation rate/min
450
350
250
150
50
-50
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
Time in minutes
control
saline
0.01mg/kg
0.25mg/kg
0.5mg/kg

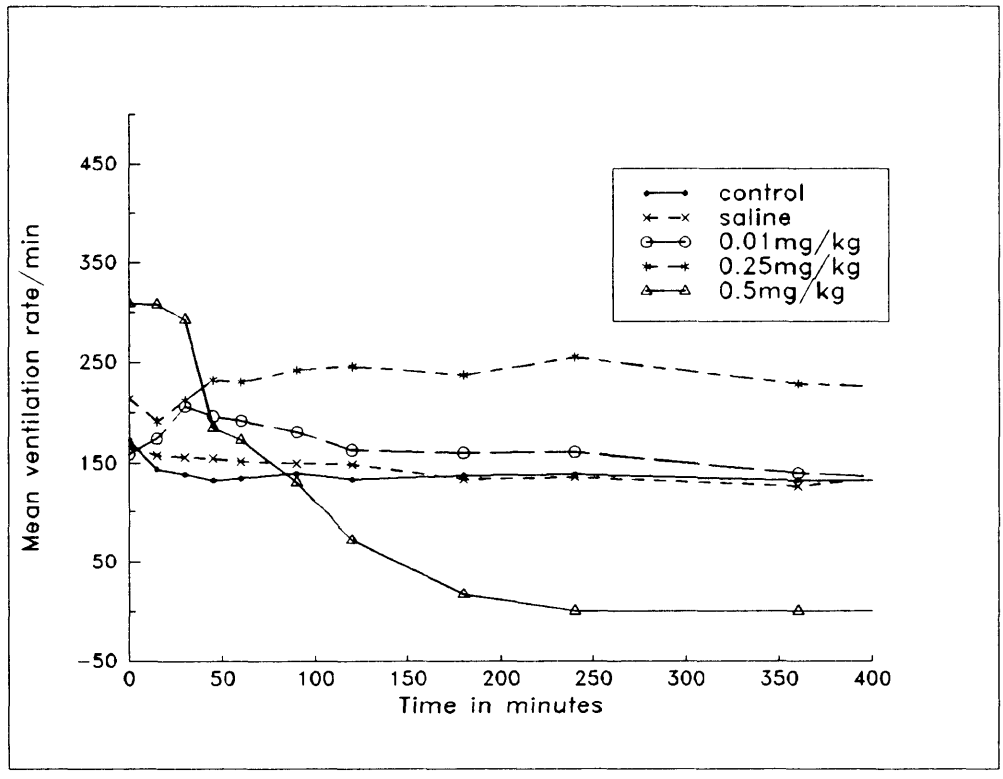
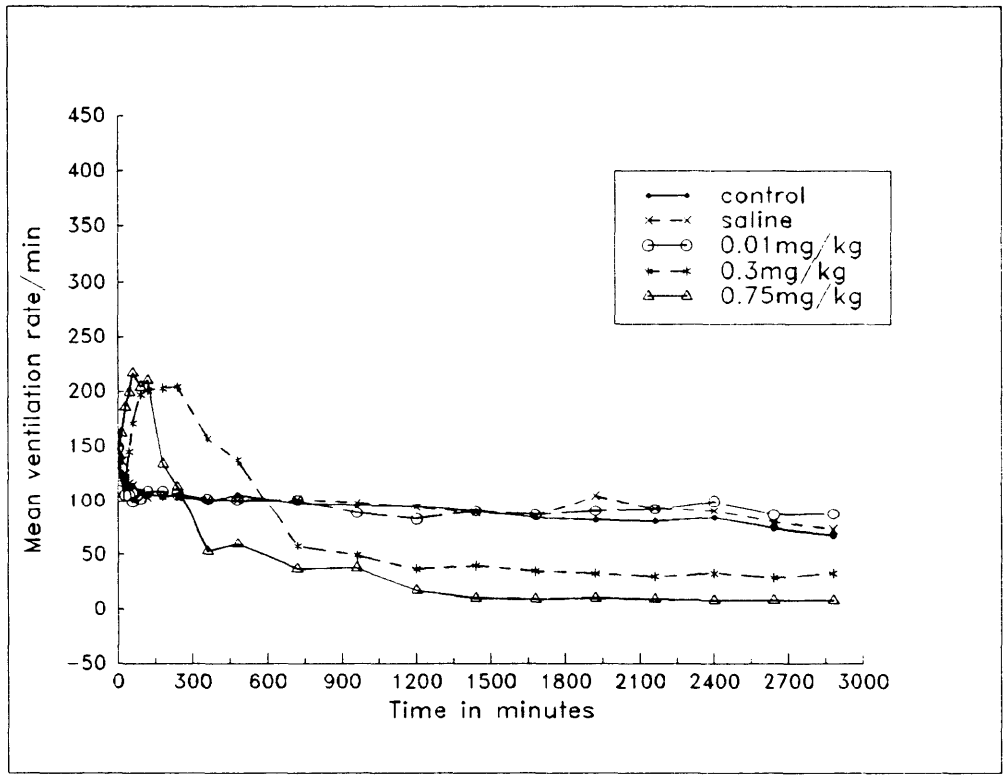

Mean ventilation rate/min
450
350
250
150
50
-50
0
50
100
150
200
250
300
350
400
Time in minutes
control
saline
0.01mg/kg
0.25mg/kg
0.5mg/kg

Figure 5.3: Mean ventilation rates / minute for *Dascyllus aruanus* in five different experimental sets over 2800 minutes (n = the total number of fish for each set).

Figure 5.4: Mean ventilation rates / minute for *Dascullus aruanus* in five different experimental sets for the first 450 minutes (n = the total number of fish for each set).


Mean ventilation rate/min
450
400
350
300
250
200
150
100
50
0
-50
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
Time in minutes
control
saline
0.01mg/kg
0.3mg/kg
0.75mg/kg

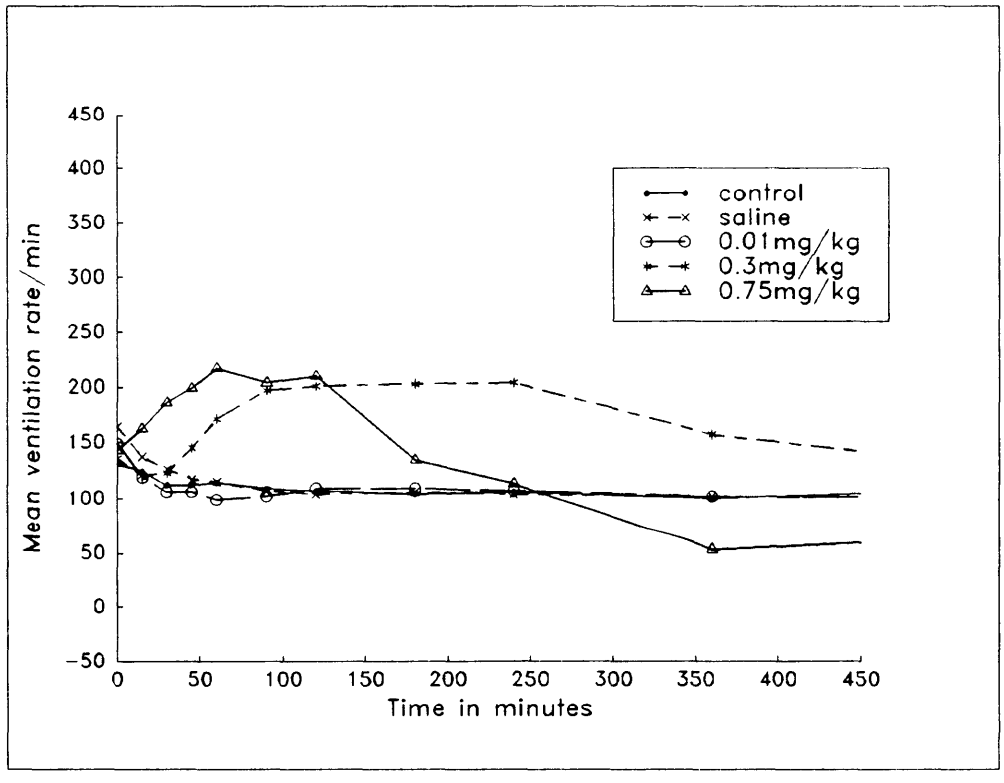
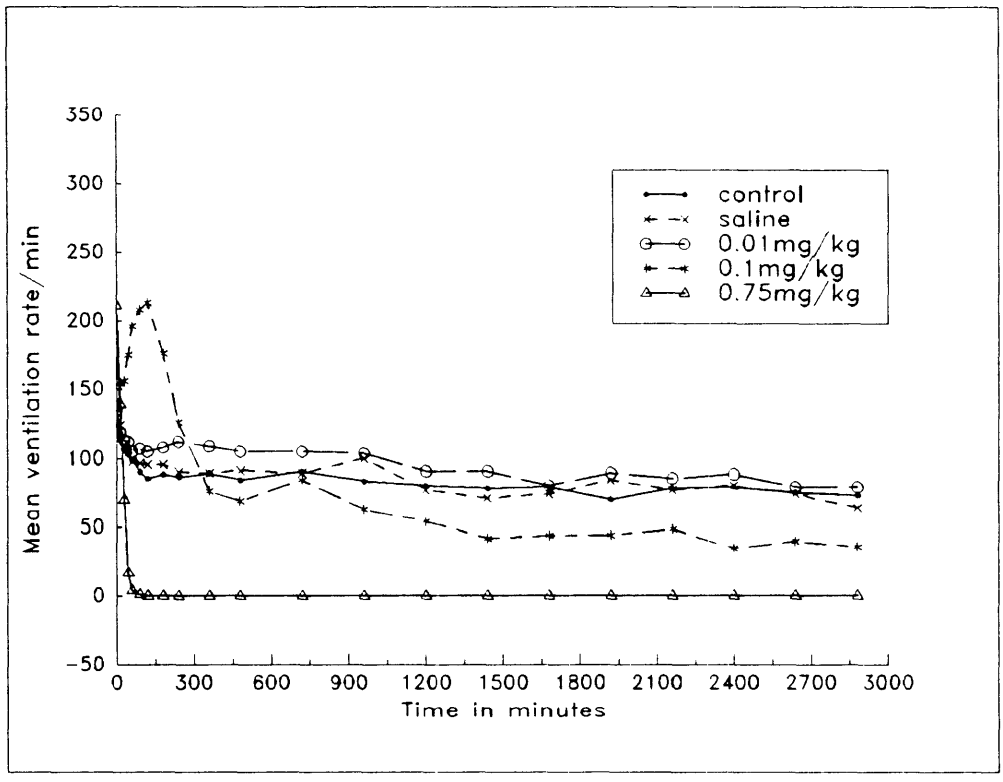

Mean ventilation rate/min
450
400
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
250
300
350
400
450
Time in minutes
control
saline
0.01mg/kg
0.3mg/kg
0.75mg/kg

Figure 5.5: Mean ventilation rates / minute for *Istiblennius meleagris* in five different experimental sets over 2880 minutes (n = the total number of fish for each set).

Figure 5.6: Mean ventilation rates / minute for *Istiblennius meleagris* in five different experimental sets for the first 450 minutes (n = the total number of fish for each set).


Mean ventilation rate/min
350
300
250
200
150
100
50
0
-50
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
Time in minutes
control
saline
0.01mg/kg
0.1mg/kg
0.75mg/kg

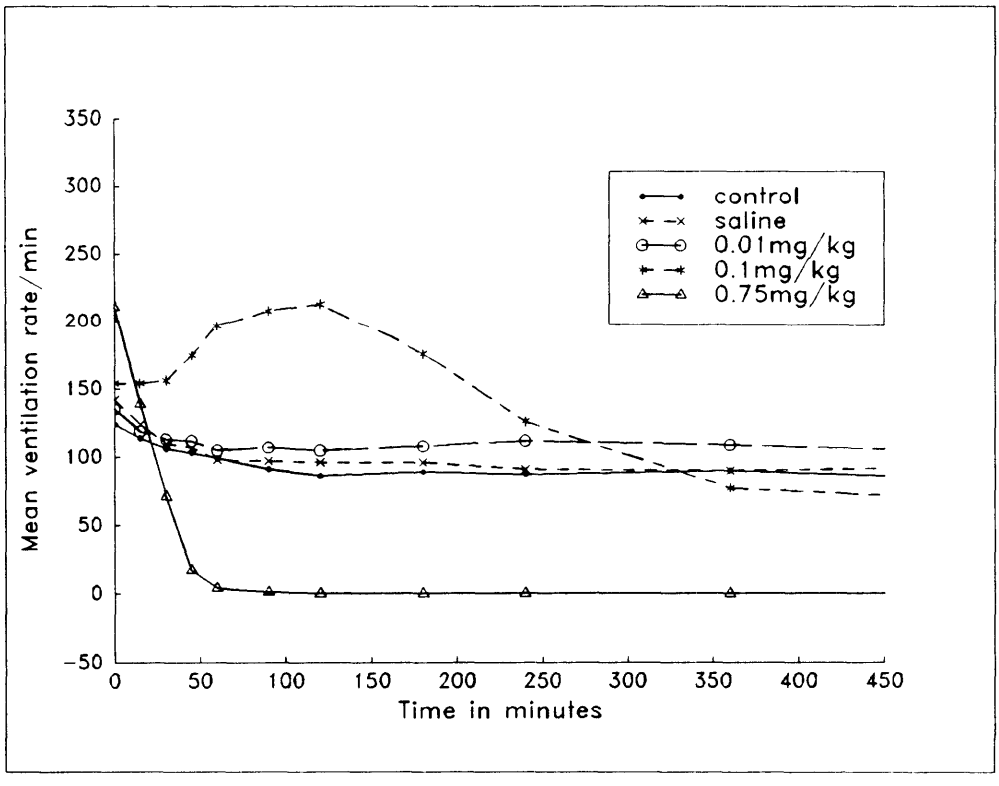
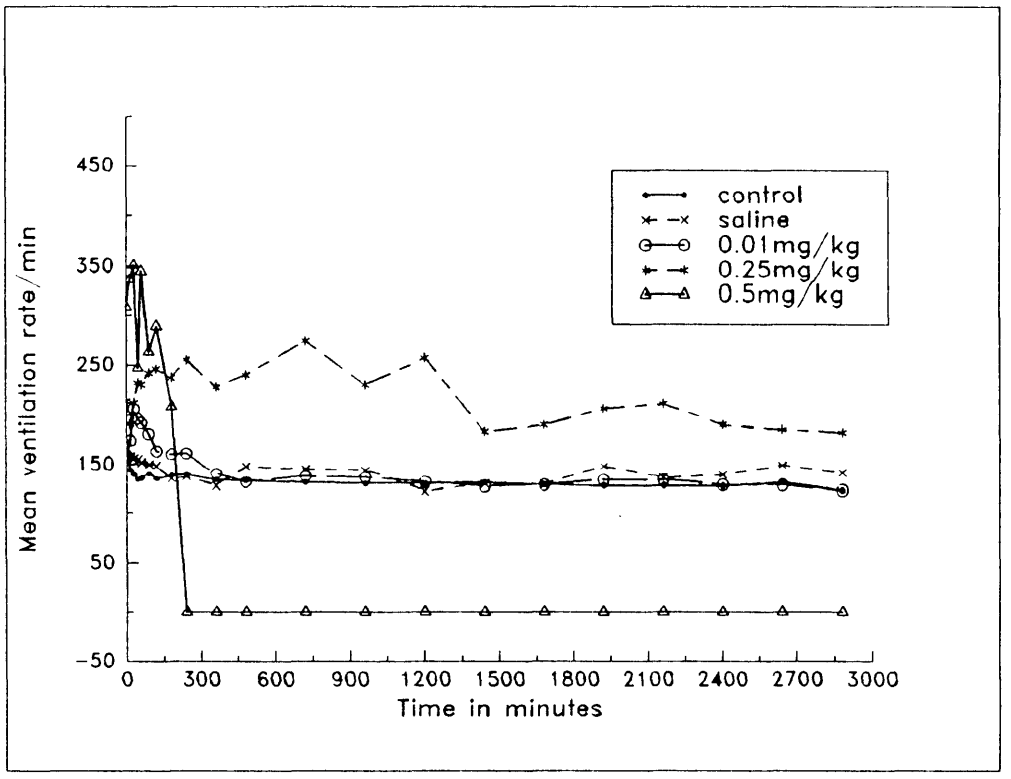

Mean ventilation rate/min
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
250
300
350
400
450
Time in minutes
control
saline
0.01mg/kg
0.1mg/kg
0.75mg/kg

Table 5.1: Means ($\overline{X}$) and standard deviations (SD) from ventilation analysis for *Chromis nitida*, *Dascyllus aruanus* and *Istiblennius meleagris*, with medium and high venom doses for the total number of fish (n = 12, dashes = all deceased).

Time in Min.		0	15	30	45	60	90	120	180	240	360	480	720	960	1200	1440	1680	1920	2160	2400	2640	2880
C. nitida																						
Control	$\overline{X}$	173	144	139	133	135	140	134	138	139	133	132	130	129	129	130	128	127	127	127	130	123
	SD	12.9	6.5	15.6	19.1	11.2	10.4	7.9	8.5	7.9	5.5	5.5	5.5	5.5	14.9	5.4	8.6	7.1	5.9	6.3	7.0	7.0
Saline	$\overline{X}$	167	158	156	155	152	150	149	134	136	127	148	145	144	122	129	128	148	135	138	149	140
	SD	11.0	7.7	7.8	7.3	6.2	7.4	11.3	10.2	13.1	10.1	12.6	9.7	15.5	6.3	6.0	6.8	7.6	8.7	6.8	5.9	6.9
0.01	$\overline{X}$	159	174	206	196	192	181	163	160	161	140	131	137	136	131	127	128	133	133	128	128	123
	SD	13.9	28.3	24.9	19.3	11.0	22.2	17.5	19.7	23.7	14.6	15.3	13.8	6.4	8.3	7.5	7.3	8.5	6.7	5.7	6.3	5.1
0.25	$\overline{X}$	214	191	212	232	230	242	246	237	255	228	221	251	134	150	107	111	120	123	111	107	106
	SD	12.8	9.8	7.5	10.3	22.5	18.3	26.0	37.4	19.9	31.7	77.0	89.7	119.6	133.5	102.6	110.9	120.3	120.0	104.6	98.0	101.6
0.5	$\overline{X}$	309	308	293	185	173	131	72	17	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	59.3	135.2	148.5	155.2	183.9	163	131.8	60.0	–	–	–	–	–	–	–	–	–	–	–	–	–
D. aruanus																						
Control	$\overline{X}$	131	124	112	112	114	109	107	104	106	100	106	98	96	95	90	83	81	80	83	74	68
	SD	8.9	10.5	7.5	7.3	6.6	6.8	7.5	5.7	8.3	6.9	5.1	5.7	6.5	3.8	6.3	9.9	10.8	9.4	11.7	11.7	14.5
Saline	$\overline{X}$	164	137	126	117	115	107	104	106	104	102	101	102	99	94	88	85	105	93	90	79	73
	SD	18.9	18.1	14.3	15.4	11.9	13.1	8.3	7.3	4.0	10.3	13.3	11.3	18.1	8.4	9.6	15.2	7.3	10.8	17.6	18.6	15.3
0.01	$\overline{X}$	149	119	106	106	99	102	109	109	107	102	101	101	88	82	90	86	90	92	100	86	87
	SD	8.5	7.2	7.1	4.4	4.4	8.4	6.1	8.3	7.5	5.5	2.6	9.4	4.1	3.2	6.2	4.8	3.3	4.0	5.6	2.9	3.9
0.3	$\overline{X}$	136	120	123	145	171	197	201	203	204	157	137	58	49	37	40	35	33	30	33	29	33
	SD	10.5	7.4	9.1	9.4	17.2	29.4	23.4	49.6	67.3	97.0	122.0	85.9	74.0	54.8	59.0	51.4	48.8	45.5	47.4	42.9	48.6
0.75	$\overline{X}$	143	162	186	199	217	204	210	134	113	53	60	37	38	17	10	9	10	9	8	8	8
	SD	8.5	10.1	15.1	13.8	28.8	46.0	743	109.2	116.8	97.6	108.0	87.0	87.6	57.7	33.2	31.0	33.0	33.0	28.3	27.0	29.0
I. meleagris																						
Control	$\overline{X}$	124	114	106	103	99	91	86	89	87	90	85	91	84	81	79	80	71	79	80	76	74
	SD	16.5	10.7	13.8	9.5	10.7	13.2	18.1	15.1	9.6	9.0	14.8	16.8	19.1	12.1	11.3	16.5	18.3	14.7	15.0	16.1	14.3
Saline	$\overline{X}$	142	124	110	107	98	97	96	96	91	90	92	90	100	78	72	75	85	78	81	75	65
	SD	21.0	16.7	10.7	10.6	13.1	10.0	9.0	10.5	8.4	7.6	9.0	7.4	11.5	7.5	10.2	12.9	9.3	8.5	8.6	8.5	9.9
0.01	$\overline{X}$	135	119	113	112	105	107	105	108	112	109	105	105	104	91	91	81	90	86	89	80	80
	SD	12.6	10.9	9.1	6.5	4.8	9.6	5.7	8.6	9.6	9.0	7.9	6.5	12.0	14.8	11.3	11.6	9.8	7.9	8.3	7.8	10.5
0.1	$\overline{X}$	154	154	156	175	196	208	213	176	126	77	70	85	64	54	42	44	44	48	35	40	36
	SD	12.8	9.1	15.2	19.7	12.4	11.2	9.3	55.5	73.0	113.6	103.3	126.2	94.2	80.1	62.3	65.6	64.6	70.6	51.5	58.8	53.8
0.75	$\overline{X}$	211	139	71	17	4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	23.5	47.2	56.3	23.2	3.1	1.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Figure 5.7: Mean ventilation rates / minute for *Chromis nitida* in five different experimental sets over 2880 minutes (n = the total number of living fish at each time interval).

Figure 5.8: Mean ventilation rates / minute for *Chromis nitida* in five different experimental sets for the first 400 minutes (n = the total number of living fish at each time interval).


Mean ventilation rate/min
450
350
250
150
50
-50
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
Time in minutes
control
saline
0.01mg/kg
0.25mg/kg
0.5mg/kg

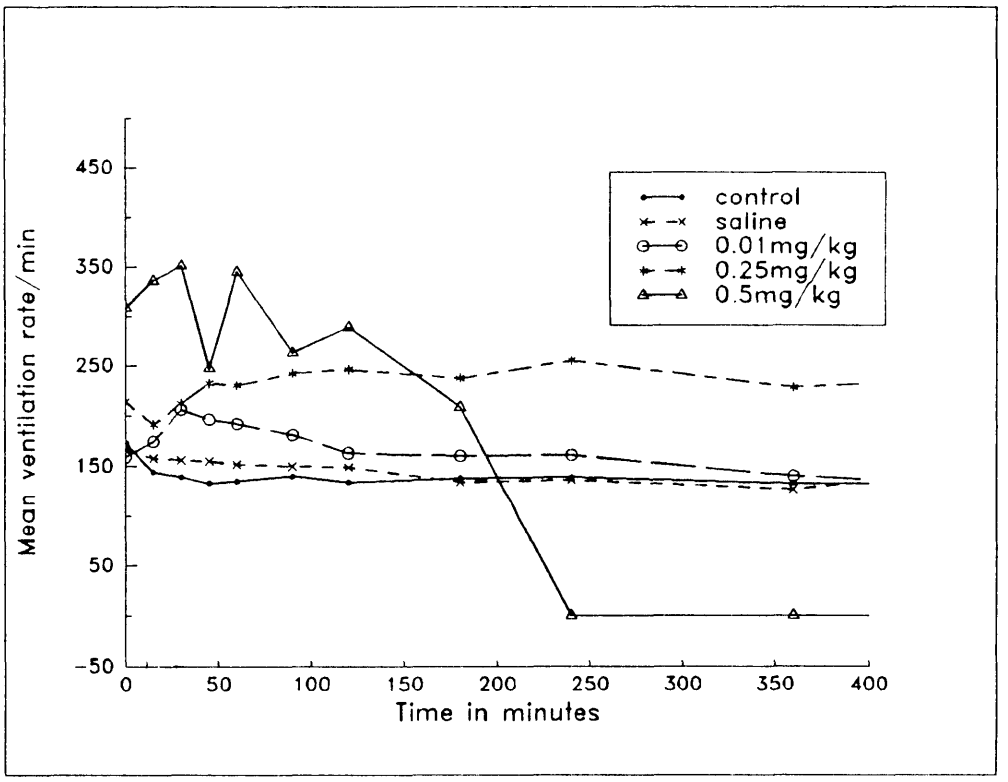
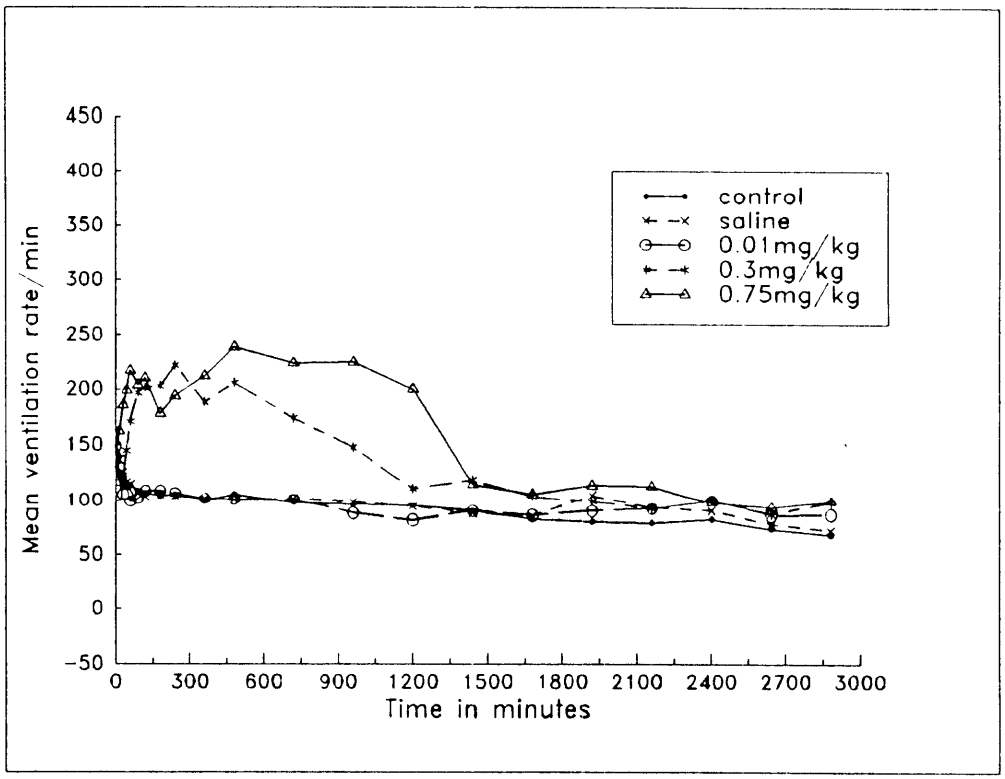

Mean ventilation rate/min
450
350
250
150
50
-50
0
50
100
150
200
250
300
350
400
Time in minutes
control
saline
0.01mg/kg
0.25mg/kg
0.5mg/kg

Figure 5.9: Mean ventilation rates / minute for *Dascyllus aruanus* in five different experimental sets over 2880 minutes (n = the total number of living fish at each time interval).

Figure 5.10: Mean ventilation rates / minute for *Dascyllus aruanus* in five different experimental sets for the first 450 minutes (n = the total number of living fish at each time interval).


Mean ventilation rate/min
450
400
350
300
250
200
150
100
50
0
-50
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
Time in minutes
control
saline
0.01mg/kg
0.3mg/kg
0.75mg/kg

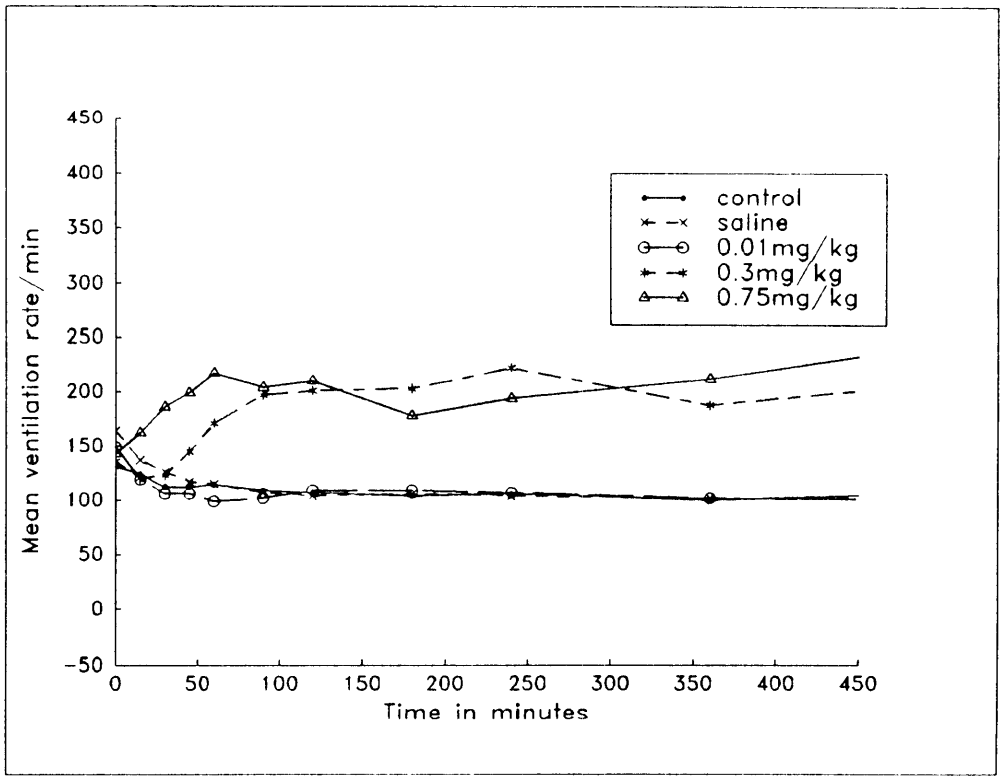
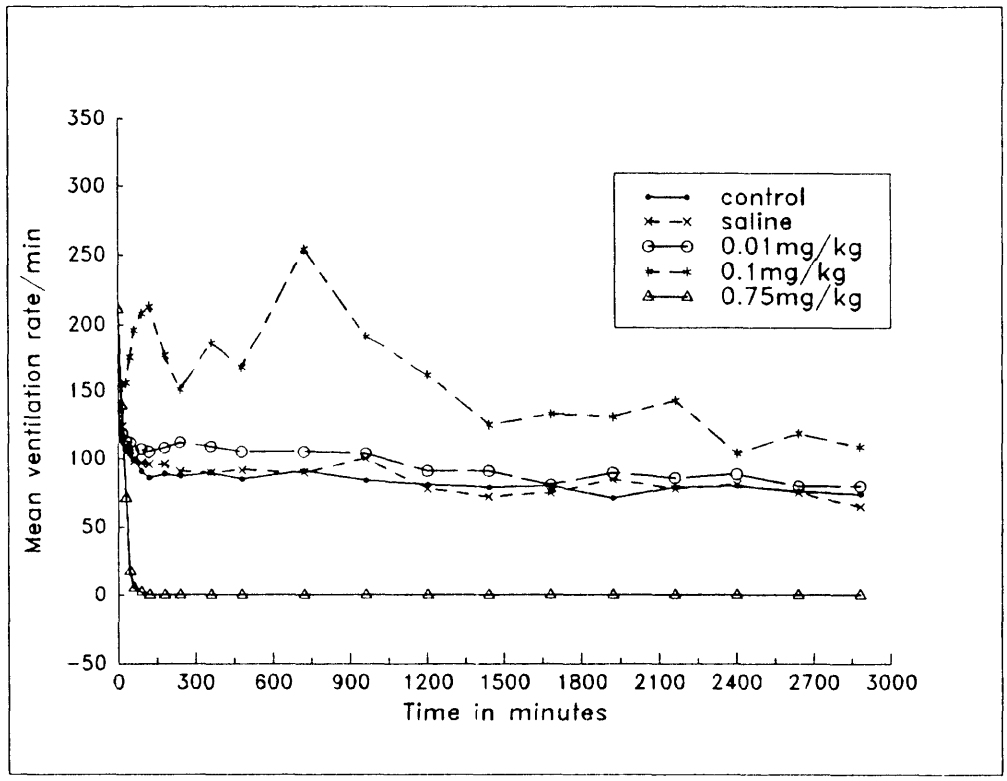

Mean ventilation rate/min
450
400
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
250
300
350
400
450
Time in minutes
control
saline
0.01mg/kg
0.3mg/kg
0.75mg/kg

Figure 5.11: Mean ventilation rates / minute for *Istiblennius meleagris* in five different experimental sets over 2880 minutes (n = the total number of living fish at each time interval).

Figure 5.12: Mean ventilation rates / minute for *Istiblennius meleagris* in five different experimental sets for the first 450 minutes (n = the total number of living fish at each time interval).


Mean ventilation rate/min
350
300
250
200
150
100
50
0
-50
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
Time in minutes
control
saline
0.01mg/kg
0.1mg/kg
0.75mg/kg

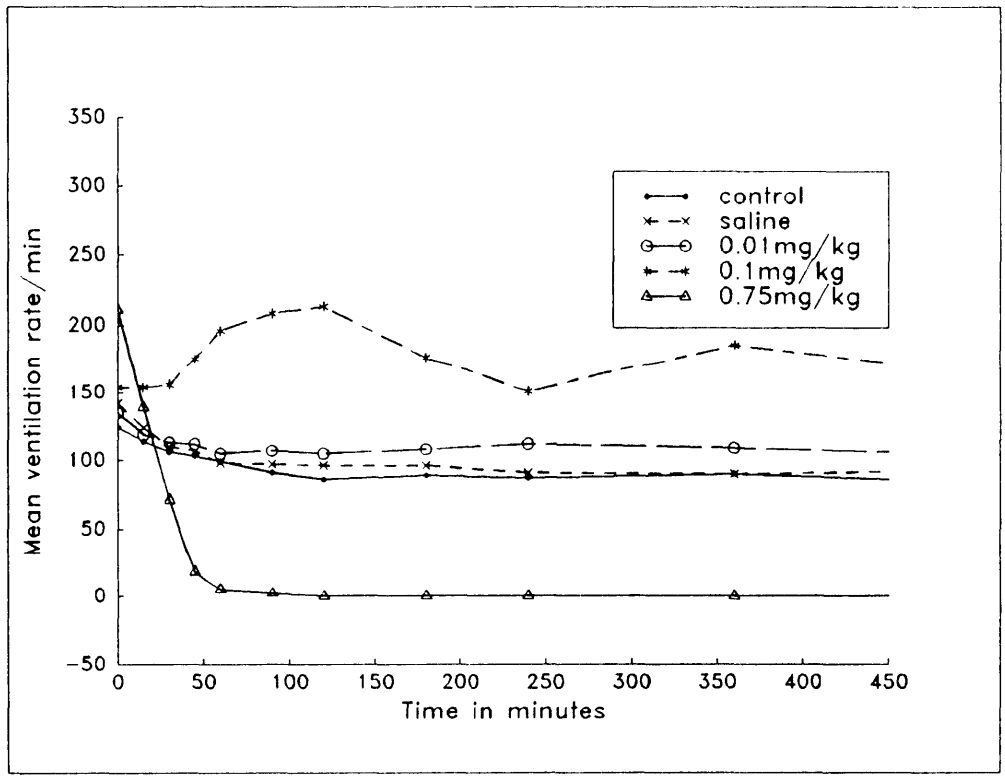

Mean ventilation rate/min
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
250
300
350
400
450
Time in minutes
control
saline
0.01mg/kg
0.1mg/kg
0.75mg/kg

Table 5.2: Means ($\overline{X}$), standard deviations (SD) and n values from ventilation analysis for *Chromis nitida*, *Dascyllus aruanus* and *Istiblennius meleagris*, with medium and high venom doses for total number of living fish (dashes = all deceased).

Time in Min.	0	15	30	45	60	90	120	180	240	360	480	720	960	1200	1440	1680	1920	2160	2400	2640	2880
C. nitida																					
0.25 $\overline{X}$	214	191	212	232	230	242	246	237	255	228	240	274	230	257	183	190	206	211	190	185	182
SD	12.8	9.8	7.5	10.3	22.5	18.3	26.0	37.4	19.9	31.7	34.9	44.7	24.9	25.1	55.6	70.6	77.1	69.4	50.8	31.7	53.9
n	12	12	12	12	12	12	12	12	12	12	11	11	7	7	7	7	7	7	7	7	7
0.5 $\overline{X}$	309	336	351	247	345	363	289	208	–	–	–	–	–	–	–	–	–	–	–	–	–
SD	59.3	98.9	64.0	126.5	53.2	131.0	42.0	0	–	–	–	–	–	–	–	–	–	–	–	–	–
n	12	11	10	9	6	6	3	1	–	–	–	–	–	–	–	–	–	–	–	–	–
D. aruanus																					
0.3 $\overline{X}$	136	120	123	145	171	197	201	203	222	188	206	174	148	111	119	104	99	92	100	87	98
SD	10.5	7.4	9.1	9.4	17.2	29.4	23.4	49.6	21.2	70.1	85.2	12.8	26.0	9.4	10.9	7.6	8.5	5.3	7.9	8.3	10.4
n	12	12	12	12	12	12	12	12	11	10	8	4	4	4	4	4	4	4	4	4	4
0.75 $\overline{X}$	143	162	186	199	217	204	210	178	194	212	239	224	225	200	115	106	114	113	98	93	99
SD	8.5	10.1	15.1	13.8	28.8	46.0	74.3	86.5	82.3	45.1	11.0	14.9	0	0	0	0	0	0	0	0	0
n	12	12	12	12	12	12	12	9	7	3	3	2	2	1	1	1	1	1	1	1	1
I. meleagris																					
0.1 $\overline{X}$	154	154	156	175	196	208	213	176	151	185	168	254	191	162	126	133	131	143	104	119	109
SD	12.8	9.1	15.2	19.7	12.4	11.2	9.3	55.5	47.7	103.0	93.5	30.5	6.2	11.4	15.6	9.8	13.0	15.6	7.9	14.0	8.1
n	12	12	12	12	12	12	12	12	10	5	5	4	4	4	4	4	4	4	4	4	4
0.75 $\overline{X}$	211	139	71	17	5	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SD	23.5	47.2	56.3	23.2	2.8	2.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
n	12	12	12	12	10	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Figure 5.13: Divisions of ventilation (see page 63) based on changes observed in ventilation rates of envenomated individuals. Used as a model for examining ventilation results of *Chromis nitida*, *Dascyllus aruanus* and *Istiblennius meleagris*.

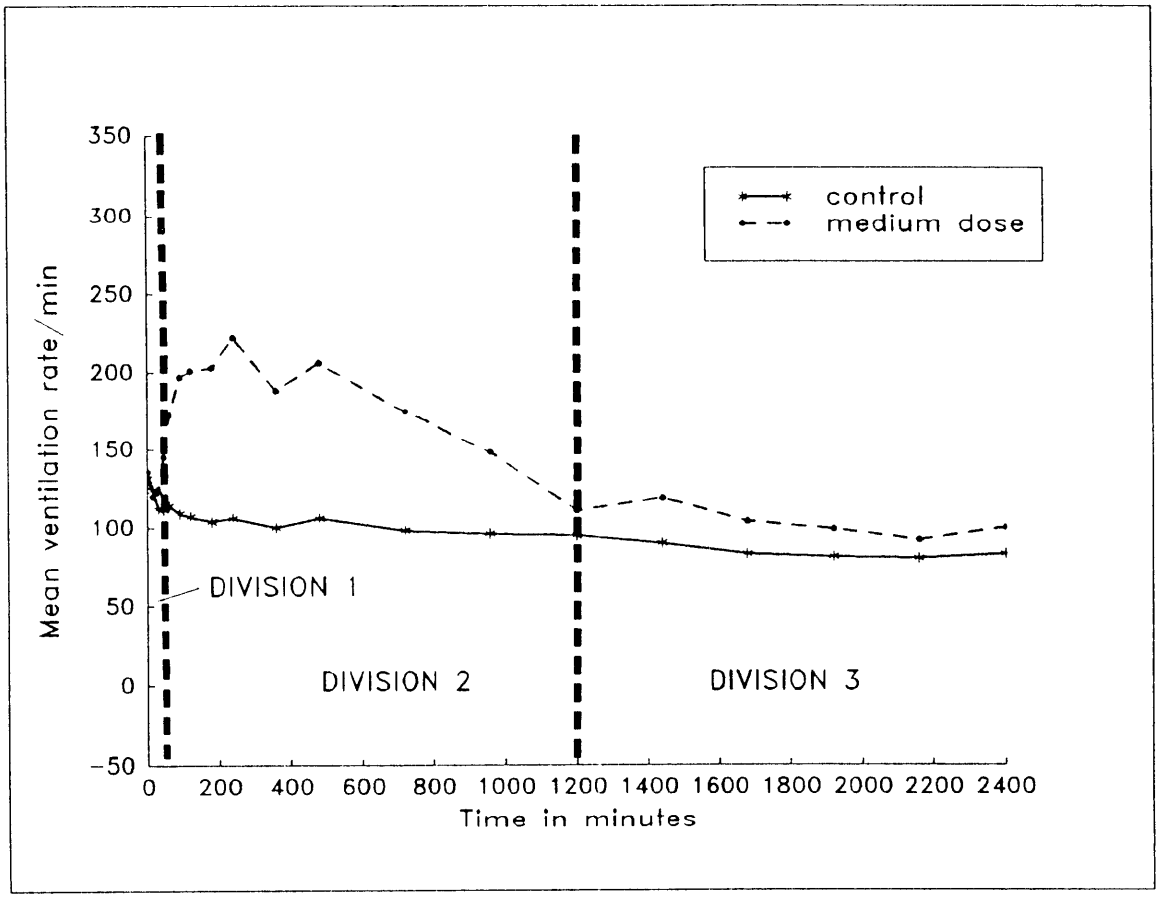

Mean ventilation rate/min
350
300
250
200
150
100
50
0
-50
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
Time in minutes
control
medium dose
DIVISION 1
DIVISION 2
DIVISION 3

Table 5.3: Comparisons from LSD calculations showing % of time that the experimental groups demonstrated no significant differences ($P > 0.05$) in ventilation rates. C – control, S – saline injected control, LD – low venom dose, MD – medium venom dose, HD – high venom dose. Parentheses refer to % of time there was no significance prior to mortality.

	C	S	LD	MD
Dascyllus aruanus				
C	–	–	–	–
S	90	–	–	–
LD	63	83	–	–
MD	8	48	41	–
HD	33*	46*	43*	93*
Istiblennius meleagris				
C	–	–	–	–
S	97	–	–	–
LD	60	84	–	–
MD	0	1	1	–
HD	0**	0**(13)	0**	0**(13)
Chromis nitida				
C	–	–	–	–
S	45	–	–	–
LD	93	54	–	–
MD	2	4	4	–
HD	0^{+}(6)	0^{+}(9)	0^{+}(6)	0^{+}(32)

* one fish survived
** dead at 120 minutes
\+ dead at 240 minutes

Chapter 6

Behavioural Studies

6.1 Introduction

Simultaneously with the ventilation studies presented in chapter 5, observations were carried out on behavioural changes in marine prey fish species envenomated with *Aipysurus laevis* venom.

The purpose of the study was:

1. to examine behavioural changes resulting from envenomation.
2. to construct a hierarchical ethogram of those changes.
3. to relate behavioural changes to changes in ventilation rates.
4. to compare inter- and intraspecific behavioural responses to envenomation.

6.2 Materials and Methods

The experimental techniques were formulated during the goldfish study (Chapter 3) and further developed during the comparative study with marine prey species (Chapter 4).

Since the behavioural studies ran in conjunction with the 48-hour ventilation study of *Chromis nitida*, *Dascyllus aruanus* and *Istiblennius meleagris* (Chapter 5), all

information regarding fish capture, maintenance, injections and handling has already been presented in section 5.2.

Based on previous quantitative and qualitative observations (Chapters 3, 4 and 5), a hierarchy of ventilatory rate and ventilatory mechanism changes were observed to exist with the experimental fish. This was also observed to be the case with the non-ventilation behavioural changes. Using the notion of an ethogram, as described by Lehner (1979), six behavioural stages were constructed, examining the ventilatory changes, as well as other accompanying behavioural changes arising from envenomation (Table 6.1). These behavioural stages were used for examining venom toxicity, which was used for descriptive and inter- and intraspecific comparative purposes. Most of the envenomated subjects demonstrated behaviours fitting the ethogram, allowing for its use throughout the study.

Behavioural observations were conducted preceeding each ventilation observation (Section 5.2), with more frequent observations required during the later, longer time periods. During each observational period, the behaviour of each fish and the time it took to enter each stage were recorded. Control and saline-injected control fish were also examined to determine if other factors besides envenomation were responsible for the behavioural changes observed in the envenomated individuals.

Graphic representations of the time for each stage change for each venom dose were constructed, employing only those fish that died from the medium and high venom doses, since they proceeded through to the final stage. Other graphic representations such as the comparison of behavioural profiles with ventilation rates, percent fish reaching each stage and the times for each fish to reach each stage are also presented.

The Fisher exact probability test (Siegel, 1956) was used to determine if there were inter- and intraspecific differences, in the total number of fish reaching each stage.

Analysis of repeated measurements, as outlined by Jennrich *et al.* (1983), was used to fit the profiles of time to stage entry and duration of each stage to a polynomial model to detect the presence of differences over time for different species and doses. This test had the necessary power at low subject numbers (Allen *et al.*, 1983; Gill, 1986). An L.S.D. program, using one-way analysis of variance, determined where

actual inter- and intraspecific differences were located.

Assessment of parallelism using G.L.I.M. (Payne, 1985) was carried out to determine if the line profiles for time to each stage possessed the same slopes and curvatures.

6.3 Results

6.3.1 Ethogram

6.3.1.1 Stage one

Stage one of the ethogram (Table 6.1) represents the initial signs of envenomation, showing fish behaviour different from that observed in the control and saline-injected control groups; the control comparisons helped rule out injection and handling procedures as affecting the behaviour. An increase in ventilatory frequency marked the onset of the stage, with coinciding activity increases and other accompanying behaviours also present. During swimming there were erratic and unpredictable movements of fins, especially of the pectoral ones. All of these movements increased with time. *I. meleagris* exhibited jerky movements of the head, mouth and fins during the initial stage of envenomation. The more sedentary nature of its habits (Moyle and Cech, 1984) were in contrast to the continous swimming of *C. nitida* and *D. aruanus* (Hughes, 1984), especially during these early behavioural stages. Occasionally fish injected with medium and high doses of venom regurgitated their food, a response also observed in a few cases during later stages. This may indicate that the venom was affecting the digestive system in some way.

6.3.1.2 Stage two

Stage two contains more serious envenomation signs. Inability to completely close the mouth and periodic exaggerated downward mouth movements were first observed during this stage, and progressively became worse with time. Leaning to one side was also observed, along with gradual loss of fin control, swimming coordination and

buoyancy control. Only the medium and high venom dose groups entered this stage, with recoveries occurring in some fish.

6.3.1.3 Stage three

More advanced signs of envenomation were observed in stage three, with a decrease in ventilatory frequency marking its beginning. An increase in ventilatory stroke volume followed (Section 1.3), seen as an exaggerated movements of the opercular shield and occasional mouth movements. Mouth movements became progressively less involved in ventilation, with reduced movements of opercular shields next to follow. Cessation of the regular movements of the opercular shields marked the end of this stage and the beginning of stage four. During stage three all fish were lying on the bottom or against the side of the aquarium as the result of total loss of swimming coordination and fin control. Attempts to maintain an upright position were futile. The presence of clear to white fecal material, as described in Appendix F, was first observed during stage three. Survival rates after stage three was extremely low, with progression through the remaining stages at a more rapid rate.

6.3.1.4 Stage four

No recoveries from envenomation occurred once fish entered stage four. A total loss of regular ventilatory movements denotes the beginning of this stage; only sporadic movements occurred and these progressively decreased in frequency. The opercular fringe, located on the outer edge of the opercular shield, demonstrated movements, as if to compensate for the loss of mouth and opercular shield movement. The fringe was the last component of the ventilatory mechanism to cease functioning, marking the end of stage four.

6.3.1.5 Stage five

Stage five, contains severe signs just prior to death. A total loss of all ventilatory movements, and bodily movements resulting only from responses to external stimuli, marks the beginning of this stage. Few sporadic body movements were observed;

these were spasm-like (mainly of the fins), and of short duration. The body darkened in synchronization with the worsening conditions, probably as the result of stress. Permanent mouth extension occured in the majority of subjects, with the mouth remaining in an open position.

6.3.1.6 Stage six

In stage six no movements were observed either spontaneously or in response to stimuli, and the fish were deemed to be dead.

Not all behaviours observed during the experiment were included in the ethogram (Table 6.1), due to their infrequency and/or to lack of regular pattern. Bottom rubbing and eye swelling, as described in Appendix F, are examples. These behavioural signs were never observed in control fish, and are thought to result from envenomation.

Surviving fish, maintained beyond the 48-hour study, continued to show signs of envenomation. These were not always observed during the time of the experiment, perhaps due to low venom doses or fish resistance. Activities of these fish appeared to decrease with time demonstrating an overall lethargy. This was never observed in control fish maintained over the same time period.

6.3.2 Behavioural stage profiles

Profiles of behavioural changes based on the average time of stage entry and duration for each envenomated species, are seen in figures 6.1 to 6.3. Only those fish (medium and high dose groups) that proceeded through to stage six, were included in the averages.

All fish subjected to a low venom dose entered only stage one, and showed only minor effects, eventually returning to normal. *C. nitida* remained for the shortest time (185 minutes) in stage one (Fig. 6.1), *D. aruanus* was next at 525 minutes (Fig. 6.2) and *I. meleagris* the longest at 1036 minutes (Fig. 6.3). The latter species may have had an initial resistance to the venom.

Fish subjected to a medium venom dose stayed longer in stage one than in the later

more advanced stages. Duration of stage progressively decreased with later stages. *I. meleagris* was an exception, however, with a longer average duration of stage five, as compared to stages two through four, indicating a final resistance before death. All three species entered into stage one at approximately the same time, with *C. nitida* taking a longer time to enter the more advanced stages. *I. meleagris* was the first to enter stage six, followed by *D.aruanus* and finally *C. nitida*, which appeared to be the most resistant to the medium dose.

Fish subjected to a high venom dose demonstrated earlier behavioural responses, thus entering the stages quicker. *I. meleagris* demonstrated the quickest response during the earlier stages, with a longer duration of stage five (comparable to the medium dose), again suggesting resistance before death. *C. nitida* was the next quickest to respond, with *D. aruanus* appearing to have the greatest overall resistance at high doses.

6.3.3 Percent of fish found in each stage

The percent of envenomated fish found in each behavioural stage can be seen in figures 6.4 to 6.6. Fish administered a low venom dose reached only stage one, with the exception of a few *C. nitida* (17%) that entered stage two. All of the fish, however, completely recovered from low doses.

All fish subjected to a medium venom dose entered stage one, with a decrease in the percent found in the more advanced stages, due to fish recovery. No *C. nitida* and *D. aruanus* survived beyond stage two, and no *I. meleagris* beyond stage three.

Of the fish subjected to high venom doses, *C. nitida* and *I. meleagris* showed 100% mortality (all entered stage six), and only one *D. aruanus* survived (after stage two).

The medium dose resulted in the most varied responses by the three species. This was due to the occurrence of some survivors in the medium dose group, as compared to all fish surviving at low doses and all dying at high dose ones. *C. nitida* demonstrated the largest variation between the stages, with *D. aruanus* next followed by *I. meleagris* with the least.

The Fisher exact probability test (Siegel, 1956) was used for comparing the

numbers of fish found in each behavioural stage. No significant differences ($P > 0.05$) were observed between the numbers of fish in adjacent stages (intraspecific) or between the numbers in the same stages (interspecific) for the low and high doses. The medium dose group, however, did demonstrate a significant difference ($P < 0.01$) between *C. nitida* and *I. meleagris*, in the number of fish found in stage three, otherwise, no inter- or intraspecific differences were found.

Intraspecific examinations of the number of fish found in the non-adjacent behavioural stages showed significant differences in the medium doses between stage one and stage six for all three species of fish (*D. aruanus* and *I. meleagris* at the 0.05 level, and *C. nitida* at the the 0.005 level). Significant differences were also noted between stage one and stages three, four and five for *C. nitida* ($P < 0.05$) and for *D. aruanus* ($P < 0.005$), with difference between stages four and five for *I. meleagris* ($P < 0.05$).

D. aruanus demonstrated no significant difference ($P > 0.05$) between stage one and six at the high venom dose, even though one fish survived.

6.3.4 Analysis of time to reach each stage and stage duration

Repeated measures (Jennrich *et al.*, 1983) was carried out as a split plot over time. However, since a test of sphericity proved highly significant ($P < 0.01$) it was necessary to use a Greenhouse Geisser correction. Differences in the average time it took fish to reach each stage, and the duration of those stages were highly significant between the experimental groups ($P < 0.01$). Due to nonhomogeneity of variance, indicating dependence of the standard deviations on the means, a log transformation was used (Allen *et al.* 1983). Probability values obtained with one-way analysis of variance were used for comparisons between the respective experimental groups.

6.3.4.1 Time to reach each stage

Only minimal differences in the average time required for envenomated fish to reach each behavioural stage were noted between those fish that survived the experiment

and those that did not (Figs 6.7 to 6.9). This suggests that the degrees of resistance in the fish did not interfer with the speed of onset of signs. Subsequent analyses were carried out only on those fish that passed through all stages and eventually died.

The test of parallelism on the line profiles (Fig 6.9) demonstrated no significant differences ($P > 0.05$) in linear trends of the lines, suggesting all fish proceeded from stage one to stage six in a similar manner. The greatest differences occurred between *C. nitida* and *I. meleagris* injected with a high venom dose, and the greatest similarity was between *D. aruanus* injected with a high venom dose and *I. meleagris* with a medium dose. A significant difference ($P < 0.01$) in curvature of lines was observed, indicating differences in the speeds with which experimental groups reached stage six.

Inter- and intraspecific comparisons of experimental groups were significant 87% of the time (84%, $P < 0.01$; 3%, $P < 0.05$), indicating that the different experimental groups of fish entered the behavioural stages at significantly different times. These comparisons can be seen for each species and doses in Appendix G, with levels of significance presented.

Intraspecific comparisons show a significant difference ($P < 0.01$) between most of the groups of fish injected with either a medium or high dose of venom, with similarities between the groups occurring only during earlier stages. Species of fish injected with the same venom dose were similar during earlier stages; the greatest similarity was between *D. aruanus* and *I.meleagris* envenomated with a medium dose. *I. meleagris* envenomated with a high venom dose was the most different from other experimental groups.

Stage one demonstrated the greatest similarity among species (71% of the total similarities). The likeness, however, decreased with more advanced stages (stage two with 17%, 11% in stage three, none observed in stages four and five and only 1% in stage six). Low dose comparisons between experimental groups showed significant differences ($P < 0.01$) 78% of the time, with no apparent patterns to these comparisons.

6.3.4.2 Stage duration

As with the previous study (Section 6.3.4.1), the analysis of stage duration exhibited only minimal differences between surviving fish and those that proceeded through to stage six. Venom resistance was felt not to have interfered with the time of stage duration, and only those fish that continued through to stage six were examined. No observations for stage six were obtained, since it represented experimental completion.

Stage duration was not dose related, as there was a significant difference ($P< 0.01$) between low doses groups, yet no significant difference ($P> 0.05$) between the low and medium doses groups. No relationship, however, was noticed between the low and high dose groups.

Inter- and intraspecific comparisons for the medium and high venom dose groups of the three species revealed no significant difference ($P> 0.05$) for 73% of all comparisons of the medium dose groups, 40% of all comparisons between the medium and high dose groups and 27% of all comparisons of the high dose groups. For all of the comparisons collectively, 55% showed significant differences ($P< 0.01$).

Intraspecific comparisons revealed *D. aruanus* as having the most similarities between experimental groups with *C. nitida* the least. Adjacent stages were similar more often than were those that were further apart; *I. meleagris* showed similarities most often and *C. nitida* the least. Actual probability values were not available for tabular and graphic representation of the results, as in chapters 5 and 8, due to a discontinuation of the appropriate computer programs at the University of New England computer centre.

Tables 6.2 and 6.3 list means, standard deviations and group numbers for (1) time to initiation of each stage and (2) duration of each stage respectively. Appendix G shows the actual comparisons between the experimental groups, with levels of significance indicated.

6.3.5 Ventilation rates at different stages

Comparisons of ventilation rates and behavioural stages for *D. aruanus*, envenomated with low, medium and high venom doses, can be seen in figures 6.10 to 6.12.

Figure 6.10 shows the comparative profile for fish injected with low doses. An initial increase in ventilation rate corresponded to entry into stage one. A subsequent decrease corresponded to a recovery from stage one to normal behaviour (normal ventilation rates, swimming and body movements). Once the normal behaviours were regained they were maintained for the remainder of the experiment, indicating complete recovery.

The comparative profile for fish injected with medium doses can be seen in figure 6.11. Stage one corresponded with an increase in ventilation rate, with stages two to four demonstrating extremely high and erratic ventilations. Stage four corresponded with a steady decrease in ventilation rates, which continued progressively to stage six. Recovery, such as occurred with medium venom doses in chapter 5, did not occur in this experiment. The long duration of the high erratic ventilations (stages 2 to 4) possibly represent physiological responses compensating for the venom's effects, with the fish probably under a great deal of physiological stress at this time. Within time, however, the fish succumbed to the venom; there was a steady decrease in ventilation ending in death.

Figure 6.12 shows the comparative profile for fish envenomated with high venom doses. The venom affected ventilation rates and behaviour quicker than did lower doses, making evaluation more difficult. Stage one was reached rather quickly, with the increase in ventilation marking its initiation. The steady decrease in ventilation observed during stage two, was accompanied by rapid entry into the advanced stages. Extremely low rates were noted in stage four, and near-zero ones in stage five. No recoveries occurred and no physiological attempts to compensate for the venom's effects, as appeared with the medium dose, were observed. There was a quick ventilation increase and short levelling off period, followed by a sharp decrease in rates terminating in death.

In order to more effectively evaluate the high venom dose it would be necessary to (1) slow down the pace of the responses by slightly decreasing the dosage or (2) shorten the time intervals used for observations.

6.4 Discussion

Construction of the ethogram (Table 6.1) was helpful in identifying the behaviours and sequences of behaviours associated with envenomation of the prey species.

The stages (sampling periods) were defined by the initiation and termination of certain behaviours that were used for describing each stage; ventilation rate and mechanism changes were used for describing stages in this study. This was described previously by Altmann (1974) for sequence sampling. The behavioural changes were consistent throughout the study for the three species, with differences only occurring in the rate and duration of change for the different dose levels.

Accompanying behaviours were not always evident, due to variability in fish, as observed during the ventilation study (Chapter 5); some behaviours were masked by more severe effects. Certain behaviours seemed to be omitted by those fish injected with high venom doses, due to the quickness of the venom's actions.

The effects of *A. laevis* venom on gold fish ventilation was observed in chapter 3. They were consistent with previous findings of Gitter and deVries (1967), Lee (1971), Limpus (1978), Mebs (1978), Campbell (1979) and Chang (1979), regarding ventilation shutdown and progression of paralysis in response to hydrophiid envenomation. Given the numerous muscles involved in ventilation by fish (Ballintijn and Hughes, 1965; Shelton, 1970; Harder, 1975) (Section 1.3), and the known effects of hydrophiid venom on nerve and muscle, it was safe to assume that ventilatory disturbances would occur in the present study.

Ventilation increase, marking the beginning of stage one, coincided with an initial increase in activity, both probably resulting from the onset of stress (Norman, 1975) caused by the venom. Increased muscular activity would result in elevated oxygen requirements, (Nilsson, 1984) in turn leading to still higher ventilation rates.

Oxygen receptors in the efferent arterial bloodstream are responsible for detecting reduced oxygen tension in the blood (Nilsson, 1984). When such reductions occur (lower oxygen partial pressure), from whatever cause, there are compensatory changes of water flow across the gills (Randall *et al.*, 1981; Moyle and Cech, 1982; Nilsson, 1984). Fish such as those in the present study, that control the amount of water

entering the gills and compensate for blood-oxygen changes, are referred to as oxygen regulators (Nilsson, 1984).

Oxygen regulators possess two methods of increasing their volume of water intake (Moyle and Cech, 1982, Section 1.3): increasing ventilatory frequency (strokes / minute) and/or increasing ventilatory stroke volume (water uptake / stroke).

A probable cause for the increase in ventilatory frequency, observed in the earlier stages of envenomation, was thought to be the result of mechanical problems of the ventilatory mechanism creating an hypoxic condition. This assumption was based on observable difficulties in ventilation such as incomplete mouth closure.

Synchronization between the buccal and the opercular pumps (Shelton, 1970; Wood and Lenfant, 1979; Moyle and Cech, 1982), and the maintenance of suitable chamber pressures (Hughes and Shelton, 1958; Lagler *et al.*, 1962; Wood and Lenfant, 1979; Randall *et al.*, 1981; Moyle and Cech, 1982), as outlined in section 1.3, guarantees a continuous unidirectional flow of water. Any disturbance to this results in the loss of the positive differential pressure gradient across the gills necessary for gas exchange (Wood and Lenfant, 1979), and results in hypoxia.

During compression of the buccal chamber complete closure of the mouth (sealed upper and lower lips) is imperative for establishment of the pressure necessary for forcing water over the gills and into the opercular chambers (Hughes and Shelton, 1958; Randall *et al.*, 1981; Moyle and Cech, 1982). Incomplete mouth closure, as observed in stage two of the present study, would allow for a reflux of water back through the mouth (Randall *et al.*, 1981), thereby decreasing chamber pressure and efficiency of gas exchange. Ability to completely close the mouth and coordination of mouth movements decreased with time. The known neurotoxic components of the venom would further complicate the situation by eventually causing a decrease in ventilatory frequency through the paralysis of ventilatory muscles.

Ventilatory stroke volume subsequently increased, tracking the decrease in ventilatory frequency, and compensating for the hypoxic effect. Exaggerated movements of the mouth and opercular shields enabled the fish to take in larger quantities of water for each ventilatory stroke (Moyle and Cech, 1982), and thereby increase efficiency of gas exchange.

Eventually the mouth ceased functioning, due to the progressive venom effects. The open extended position could have resulted from hypercontraction of muscles controlling the mouth, which, according to Harris *et al.* (1980), is known to occur as the result of envenomations of this kind. This may represent possible evidence of myotoxic effects on the fish, since its occurrence was after the initial neurotoxic effects, as discussed in section 5.3.2.

With the shutdown of the buccal pump, the opercular pump acquired the responsibility of maintaining the required partial pressure gradient for gas exchange. It too, however, eventually surrendered to the effects of the venom, leaving only the fringe of the opercular shield to show the final ventilatory movement. The random movements observed after stage four were more spasm-like, contributing little to water movement over the gills and subsequent gas exchange.

An increase in carbon dioxide (hypercapnia) concurrently occurs with hypoxia (Randall *et al.*, 1981) as a consequence of decrease in ventilatory efficiency. Hypercapnia, according to Fry (1957), affects the loading of oxygen onto hemoglobin. At a certain partial pressure of carbon dioxide there is a dramatic increase in the required partial pressure of oxygen needed for the hemoglobin pickup. The progression of the paralyzing effects of the venom on the ventilatory system of the fish, further complicated the situation by not premitting increased water intake, needed to compensate for the greater respiratory gas exchange required by the the elevated activity levels.

Once a reduction in the partial pressure of oxygen becomes low enough to reduce metabolism a decrease in activity occurs (Fry, 1957). Such a decrease in activity was noted to become progressively worse as this study proceeded. When blood-oxygen becomes low enough in fish, according to Fry (1957), activity is reduced to bare maintenance activities, with no performance of external work. By stage three, all fish were lying on the bottom of the aquarium, with minimal movement, decreasing to no movement or just spasm-like movements by stage five. When the partial pressure of oxygen is low enough death rapidly follows. This was indicated by a sudden decrease in ventilation rate and a rapid progression to stage six (Figs. 6.11 and 6.12).

Inability to maintain swimming coordination and an upright position may be the result of loss of an equilibrium sense in combination with the lack of fin control, the

latter arising from the paralyzing effects of the venom's neurotoxic components.

All organisms (Budelmann, 1988) with locomotor abilities possess an equilibrium receptor system enabeling them to control position and motor activities using gravity as a reference (Budelmann, 1988; Platt, 1988). All vertebrates, according to Platt (1988), possess a mechanoreceptor within the inner ear, which when stimulated by changes of an overlying fluid (or mass), allows for recognition of position change and subsequent corrections if necessary. Possible venom neurotoxic effects (Section 1.1) on the acetyl choline synapses (Platt, 1988) of the nerves serving this mechanism, could prevent proper recognition of equilibrium changes, thereby preventing necessary adjustments to be made. In the present study, fin involvement through progressive loss of fin movement and control, resulted from the paralyzing effects of venom.

Fins are controlled by radial muscles (Alexander, 1967) served by spinal nerve branches. By changing muscle tonus, fin stiffness and position can be altered, allowing for control of swimming, maneuvering, hovering in midwater and maintaining body position. According to Webb (1978), symmetrical movements of paired fins cancel tendencies to pitch and roll and allows for control of body positioning and swimming; asymmetrical fin movements are employed for maneuvering. Median fins are also used for maneuvering, with the caudal fin used by many teleosts for hovering and swimming (Alexander, 1967). Pomacentridae (*C. nitida* and *D. aruanus*) possess a combination of pectoral and caudal fin propulsion, according to Lindsey (1978), with the Blinadea (*I. meleagris*) using an undulation of the body in combination with the pectoral fins for propulsion. Being bottom dwellers the latter are not as dependent on their fins for body positioning.

The progression of fin paralysis (anterior to posterior) helps explain the orderly occurrence of behavioural signs, as seen in stages two and three. The inability to maintain an upright position and to prevent roll from occurring, was due to paralysis of the pectoral fins, with the loss of maneuvering, hovering and swimming control, caused by paralysis of the pectoral, median and caudal fins.

The swimbladder, which corrects for density and helps maintain position at a desired depth (Alexander, 1967; Moyle and Cech, 1982), is not responsible for maintaining proper body equilibrium and posture at that depth. According to Alexander

(1967), this is the responsibility of the fins. A loss of equilibrium or body position causes a change in buoyancy, independent of swimbladder involvement, and can only be corrected by the fins. Since body orientation did change in this study, as the result of fin paralysis, fish sank to the bottom due to their inability to compensate adequately for loss of buoyancy control.

To further support the idea that fin paralysis is responsible for the observed swimming and body orientation problems, a pilot study (unpublished by author) was carried out examining selective fin amputations on control fish. The fish responded in a way similar to envenomated fish, and in agreement with Alexander (1967) and Webb (1978).

The inter- and intraspecific graphic and statistical comparisons failed to show any major differences in patterns of resistance or receptivity to venom. The occurrence of mortalities was observed in all three species with differences on how fish responded to the envenomations reflecting their lifestyles and habitat types.

The major difference appeared to be between the pomacentrids (*C. nitida* and *D.aruanus*) and *I. meleagris*, even though the overall responses to envenomations were in agreement for all three species. According to Moyle and Cech (1982), oxygen requirements are lower for *I. meleagris* than for the pomacentrids, due to their sedentary and secretive nature (Graham, 1976; Moyle and Cech, 1982; Hughes, 1984). The former has cutaneous respiration (Graham, 1976), which would partially compensate for loss of ventilation of the gills and may help explain some of its resistance to venom, especially during stage five. Variation in ventilation rates were expected, which, according to Norman (1975), vary from species to species in relation to metabolic levels and habitat types.

All of the envenomated fish entered stage one of the ethogram, with 100% survival for those receiving the low dose. There were survivors in all three species at the medium dosage but 100% mortality (except for one *D. aruanus*) at the high one.

Regarding fish number entering each stage, the only significant difference was observed between non-adjacent stages of the medium venom dose. This result reflects survivorship at this dose, with a significant number of fish that entered the earlier stages surviving.

The test of parallelism demonstrated no differences in behavioural profiles, during progression from stage one to stage six. The only differences were in the curvatures of the lines, indicating differences in total time to death. These comparisons demonstrate that the species react to the venom at different speeds, but in the same general manner.

Comparisons of time of entry into each stage were similar in earlier stages. This decreased in time, however, possibly as the result of different long-term responses to the venom by the three species.

No apparent patterns of significance could be determined in stage duration, and similarities were greatest between intraspecific adjacent stages, as compared to the non-adjacent stages. High degrees of similarities occurred between the medium dose groups; those fish initally appeared to be resistant, but eventually succumbed to the venom.

Ventilation rates and behavioural signs can be used as indicators of severity of envenomation. Slightly elevated ventilation rates in conjuction with behaviours characteristic of stage one would indicate a mild envenomation. A higher increase in ventilation rate, with an erratic profile, accompanied by more advanced behavioural signs of envenomation, would indicate a moderate envenomation. The higher dose was easily recognized by the rapid onset of the behavioural signs, as well as an increase and subsequent decrease in ventilation rates.

The crossover point for the ventilation rates and behavioural signs (at the medium and high doses) is the point where the subject will subsequently die. This point was reached more rapidly at high doses.

Perhaps information on crossover points and severity of envenomation can be used as a means of examining pharmacological and physiological interactions more closely. By finding out more about venom, possibly the impact of envenomation on humans can be reduced.

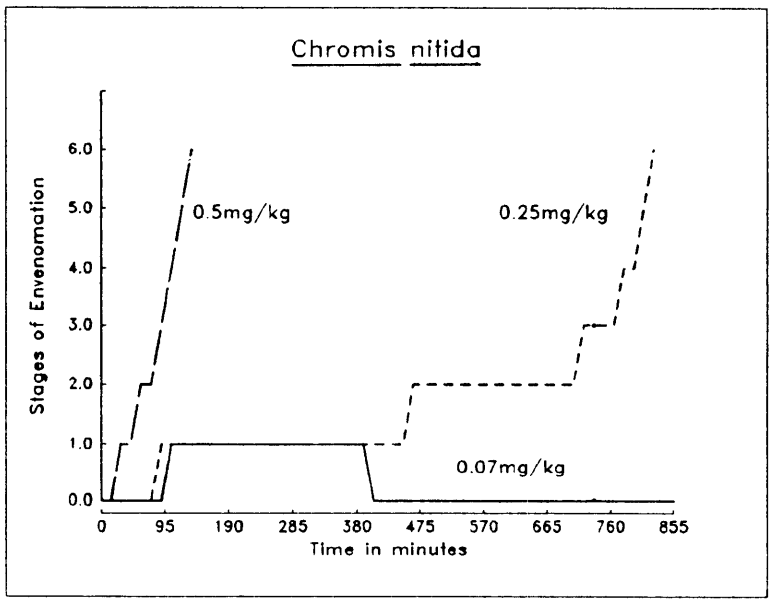
Table 6.1: Stages and signs of behavioural changes for marine fish envenomated with *Aipysurus laevis* venom.

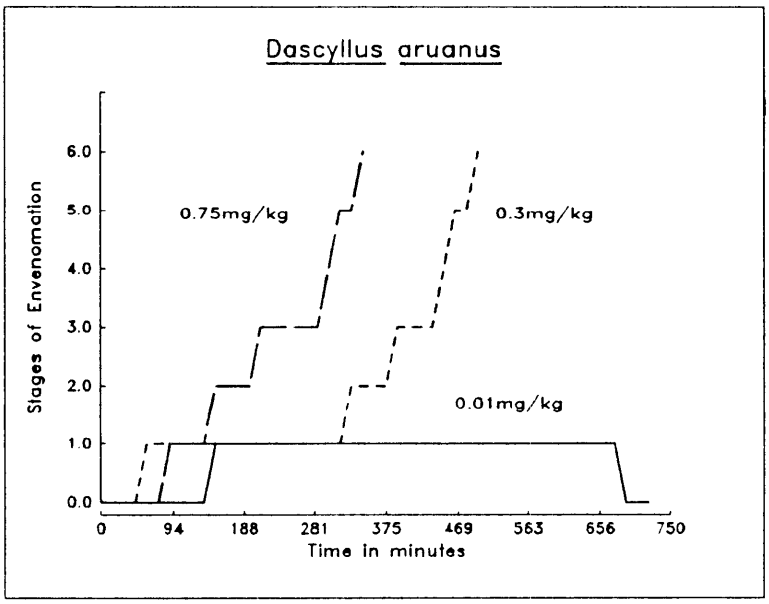
Stage One: *Initial Signs* A Increase in ventilatory frequency B Increase in activity C Regurgitation of food
Stage Two: *Secondary Signs* A Lack of complete mouth closure B Periodic exaggerated downward mouth extension C Leaning to one side D Loss of fin control E Loss of swimming coordination F Buoyancy-like problems
Stage Three: *Advanced Signs* A Decrease in ventilatory frequency B Increase in ventilatory stroke volume (exaggerated opercular shield movements) C Laying on bottom or against aquarium side D Start of sporadic uncontrolable swimming swift, with nondirectional bursts E Presence of white to clear fecal material
Stage Four: *Serious Signs* A Total loss of regular ventilatory rates with only sporadic movements B Opercular fringe movement
Stage Five: *Pre-Mortality Signs* A Total loss of all ventilatory movements, respond to stimuli, however B Occasional sporadic movements C Occasional uncontrolable fin movement sometimes gentle other times spasm-like D Color change in body occurs, becoming darker as signs worsen E Permanent mouth extension
Stage Six: *Death* A No movements of any type B No response to stimuli

Figure 6.1: Behavioural profiles of *Chromis nitida* showing time of stage entry and duration for a low, medium and high dose of *Aipysurus laevis* venom.

Figure 6.2: Behavioural profiles of *Dascyllus aruanus* showing time of stage entry and duration for a low, medium and high venom dose of *Aipysurus laevis* venom.

Figure 6.3: Behavioural profiles of *Istiblennius meleagris* showing time of stage entry and duration for a low, medium and high dose of *Aipysurus laevis* venom.


Chromis nitida
Stages of Envenomation
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.5mg/kg
0.25mg/kg
0.07mg/kg
0
95
190
285
380
475
570
665
760
855
Time in minutes


Dascyllus aruanus
Stages of Envenomation
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.75mg/kg
0.3mg/kg
0.01mg/kg
0
94
188
281
375
469
563
656
750
Time in minutes

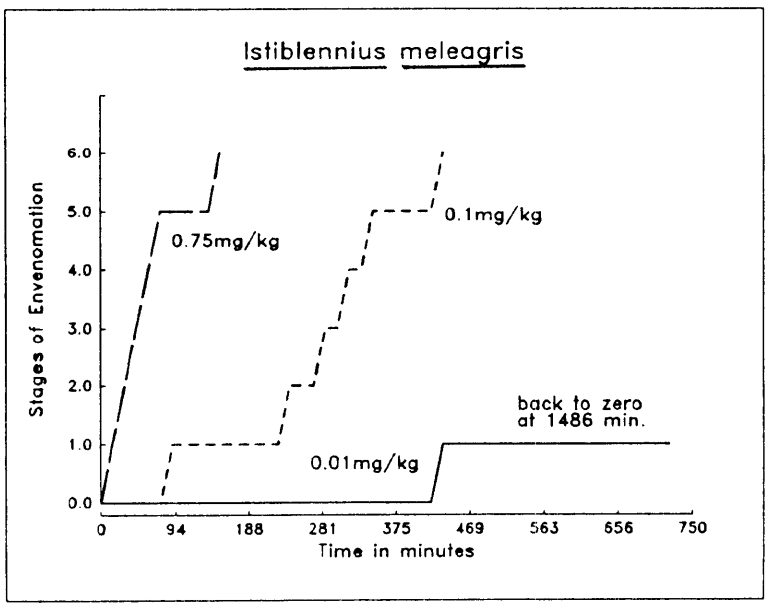
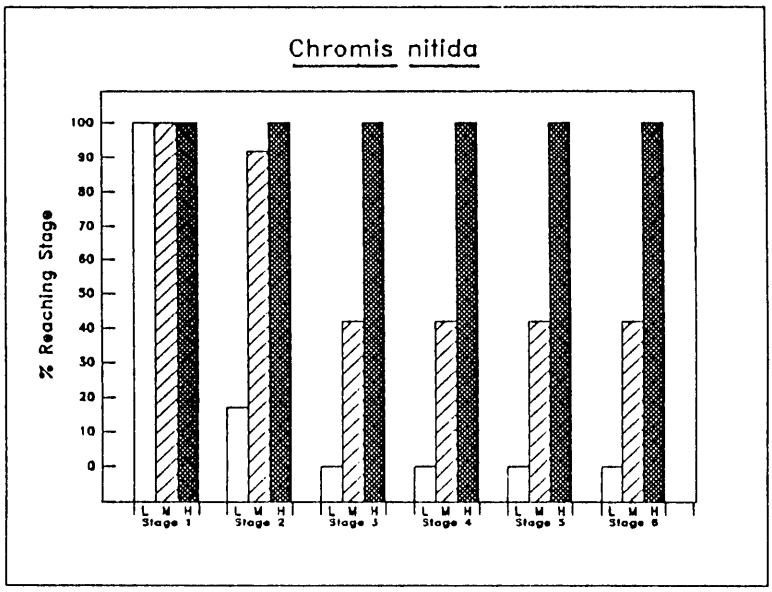
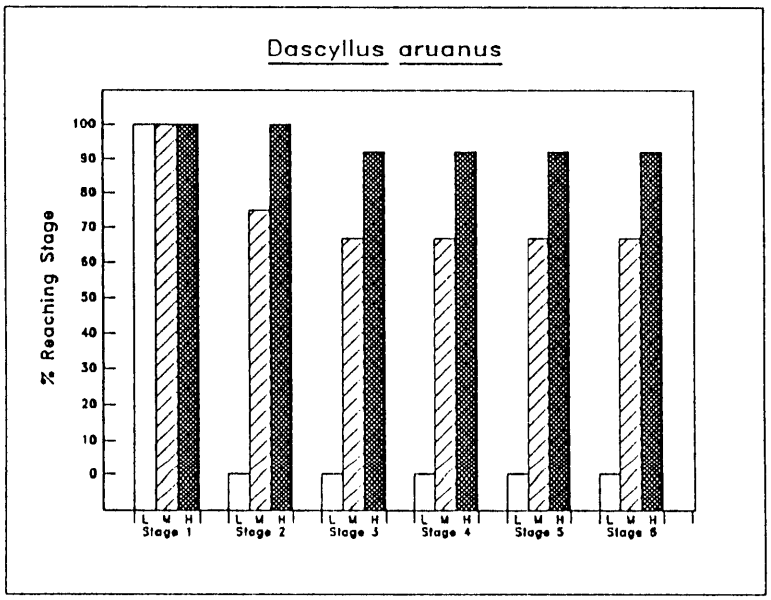

Istiblennius meleagris
Stages of Envenomation
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.75mg/kg
0.1mg/kg
back to zero
at 1486 min.
0.01mg/kg
0
94
188
281
375
469
563
656
750
Time in minutes

Figure 6.4: Percent of envenomated *Chromis nitida* that reached each stage for a low (L), medium (M) and high (H) dose of *Aipysurus laevis* venom.

Figure 6.5: Percent of envenomated *Dascyllus aruanus* that reached each stage for a low (L), medium (M) and high (H) dose of *Aipysurus laevis* venom.

Figure 6.6: Percent of envenomated *Istiblennius meleagris* that reached each stage for a low (L), medium (M) and high (H) dose of *Aipysurus laevis* venom.


Chromis nitida
% Reaching Stage
100
90
80
70
60
50
40
30
20
10
0
L M H
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Stage 6


Dascyllus aruanus
% Reaching Stage
100
90
80
70
60
50
40
30
20
10
0
L M H
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Stage 6

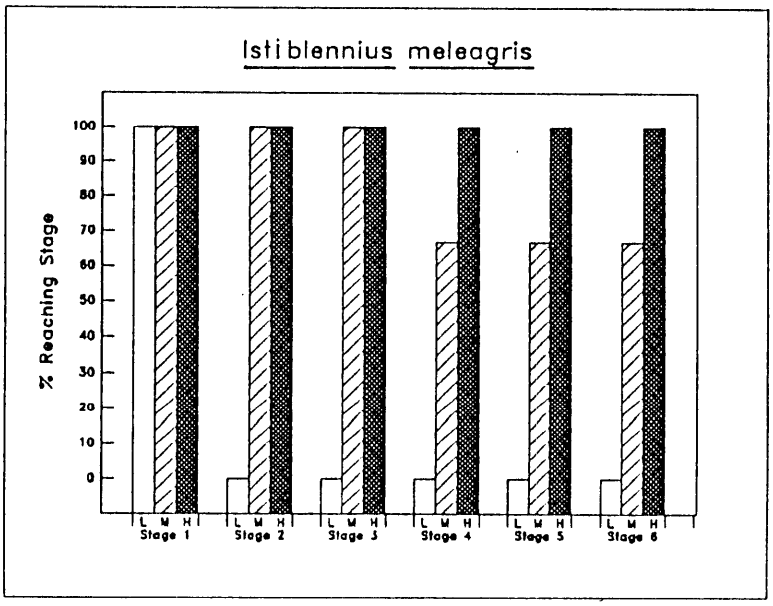
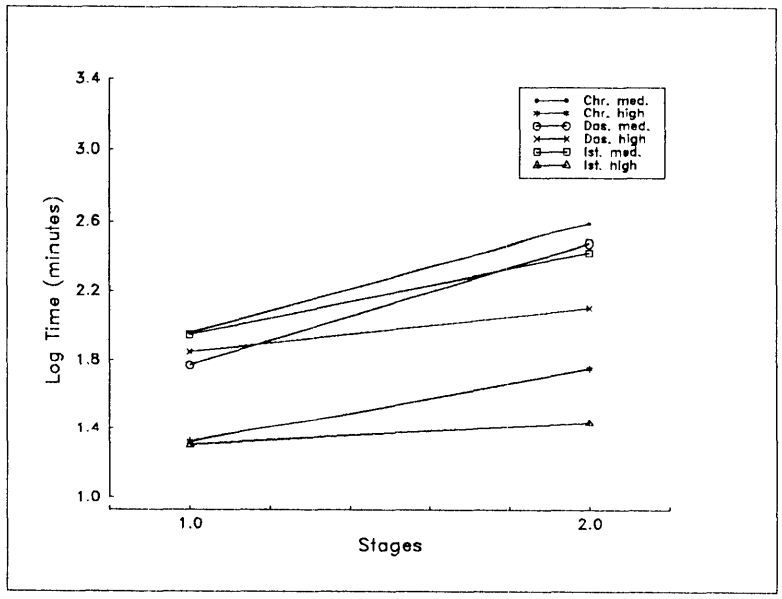
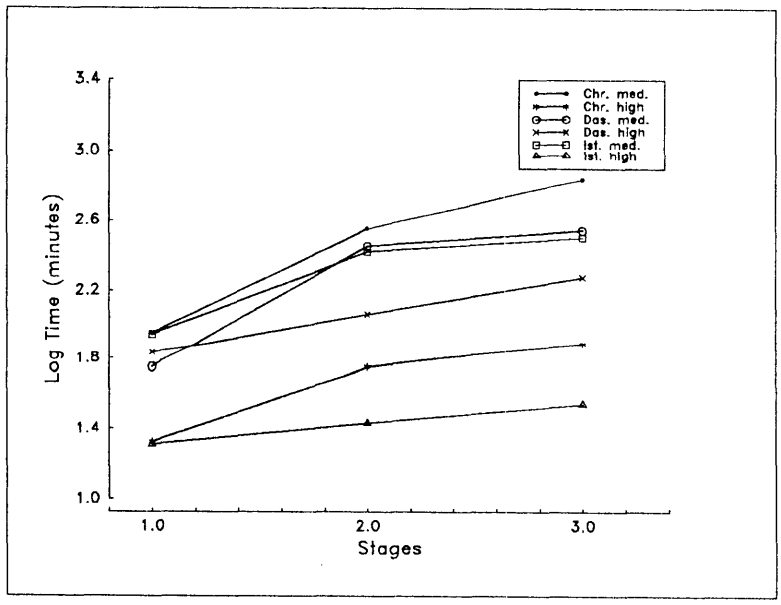

Istiblennius meleagris
% Reaching Stage
100
90
80
70
60
50
40
30
20
10
0
L M H
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Stage 6

Figure 6.7: Behavioural line profiles for *Chromis nitida* (Chr.), *Dascyllus aruanus* (Das.) and *Istiblennius meleagris* (Ist.) for a medium (med.) and high dose of *Aipysurus laevis* venom, proceeding through to stage two.

Figure 6.8: Behavioural line profiles for *Chromis nitida* (Chr.), *Dascyllus aruanus* (Das.) and *Istiblennius meleagris* (Ist.) for a medium (med.) and high dose of *Aipysurus laevis* venom, proceeding through to stage three.

Figure 6.9: Behavioural line profiles for *Chromis nitida* (Chr.), *Dascyllus aruanus* (Das.) and *Istiblennius meleagris* (Ist.) for a medium (med.) and high dose of *Aipysurus laevis* venom, proceeding through to stage six.


Chr. med.
Chr. high
Das. med.
Das. high
Ist. med.
Ist. high
Log Time (minutes)
3.4
3.0
2.6
2.2
1.8
1.4
1.0
1.0
2.0
Stages


Chr. med.
Chr. high
Das. med.
Das. high
Ist. med.
Ist. high
Log Time (minutes)
3.4
3.0
2.6
2.2
1.8
1.4
1.0
1.0
2.0
3.0
Stages

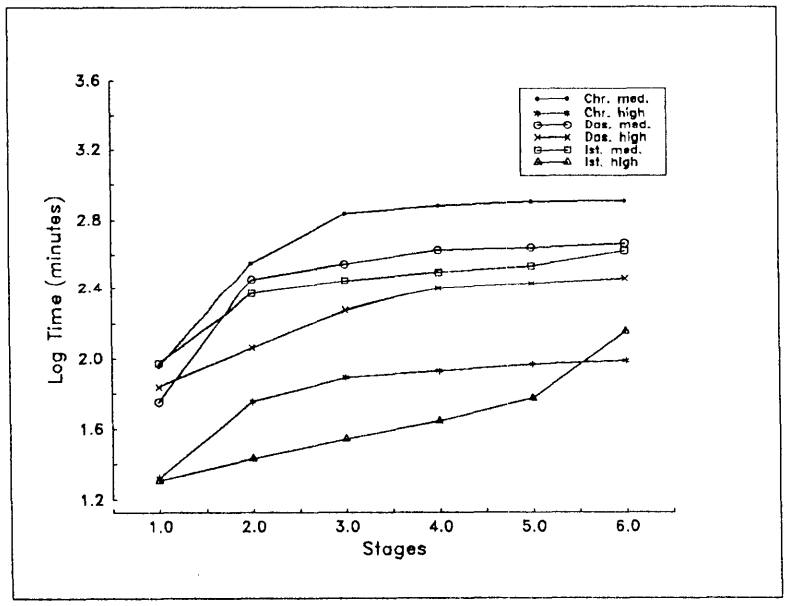

Chr. med.
Chr. high
Das. med.
Das. high
Ist. med.
Ist. high
Log Time (minutes)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
1.0
2.0
3.0
4.0
5.0
6.0
Stages

Table 6.2: Mean values ($\overline{X}$) for the analysis of time to each stage (in minutes) with corresponding standard deviations (SD) and numbers of individuals (n), for those fish that proceeded from Stage 1 to Stage 6. CNM – *Chromis nitida* medium dose; CNH – *Chromis nitida* high dose; DAM – *Dascyllus aruanus* medium dose; DAH – *Dascyllus aruanus* high dose; IMM – *Istiblennius meleagris* medium dose; IMH – *Istiblennius meleagris* high dose.

		CNM	CNH	DAM	DAH	IMM	IMH
Stage 1	$\overline{X}$	90	21	57	69	94	20
	SD	1.1	1.9	1.3	1.6	1.2	1.4
	n	5	12	8	11	8	12
Stage 2	$\overline{X}$	356	57	285	116	232	27
	SD	2.3	1.7	1.8	1.6	1.2	1.3
	n	5	12	8	11	8	12
Stage 3	$\overline{X}$	688	78	350	189	281	35
	SD	1.5	1.6	1.6	1.6	1.1	1.2
	n	5	12	8	11	8	12
Stage 4	$\overline{X}$	756	85	426	248	315	44
	SD	1.4	1.7	1.5	1.8	1.2	1.2
	n	5	12	8	11	8	12
Stage 5	$\overline{X}$	794	92	438	268	341	59
	SD	1.3	1.7	1.5	1.8	1.2	1.3
	n	5	12	8	11	8	12
Stage 6	$\overline{X}$	804	97	465	290	419	142
	SD	1.3	1.7	1.4	1.7	1.3	1.4
	n	5	12	8	11	8	12

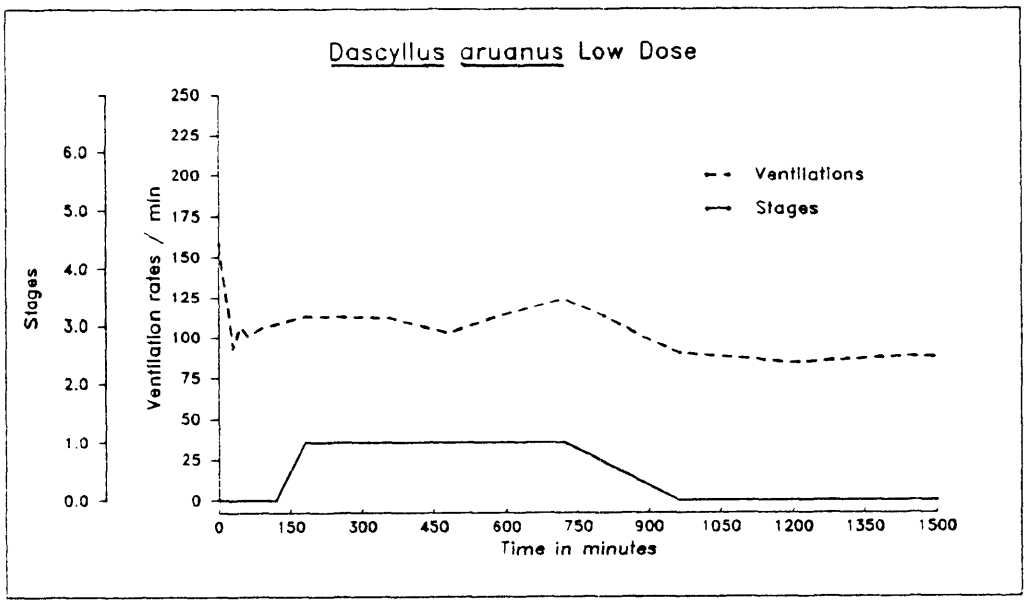
Table 6.3: Mean values ($\overline{X}$) for the analysis of time in each stage (in minutes) with corresponding standard deviations (SD) and numbers of individuals (n), for those fish that proceeded from Stage 1 to Stage 6. CNM – *Chromis nitida* medium dose; CNH – *Chromis nitida* high dose; DAM – *Dascyllus aruanus* medium dose; DAH – *Dascyllus aruanus* high dose; IMM – *Istiblennius meleagris* medium dose; IMH – *Istiblennius meleagris* high dose.

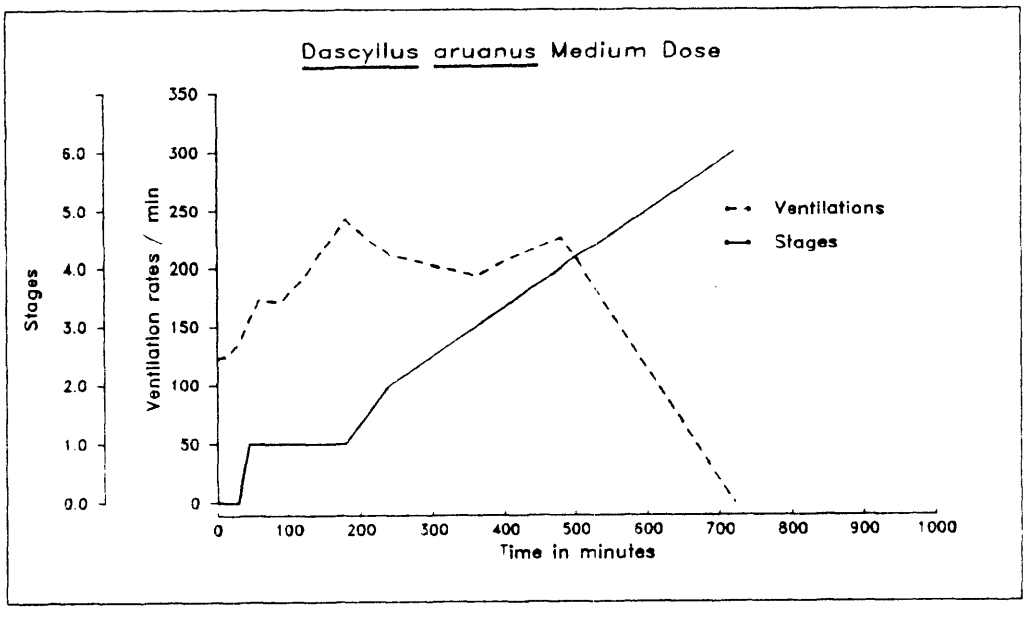
		CNM	CNH	DAM	DAH	IMM	IMH
Stage 1	$\overline{X}$	180	33	215	38	133	6
	SD	5.9	1.8	2.2	2.0	1.4	1.7
	n	5	12	8	11	8	12
Stage 2	$\overline{X}$	139	20	28	61	37	7
	SD	5.1	2.1	4.6	2.2	2.1	1.7
	n	5	12	8	11	8	12
Stage 3	$\overline{X}$	31	6	52	42	28	8
	SD	4.1	2.8	2.2	3.3	2.0	1.9
	n	5	12	8	11	8	12
Stage 4	$\overline{X}$	16	6	11	14	20	11
	SD	2.6	2.9	2.0	1.8	2.2	2.7
	n	5	12	8	11	8	12
Stage 5	$\overline{X}$	5	4	23	17	65	78
	SD	2.9	1.7	1.4	2.0	2.4	1.7
	n	5	12	8	11	8	12

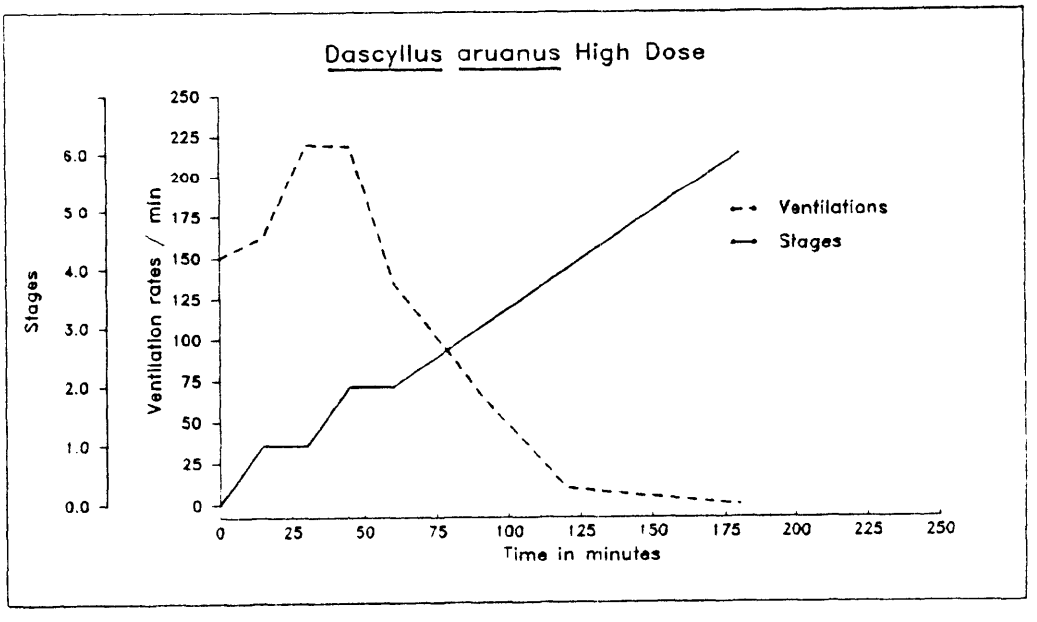
Figure 6.10: Comparison of ventilation rates and behavioural profile for *Dascyllus aruanus* envenomated with a low dose of *Aipysurus laevis* venom.

Figure 6.11: Comparison of ventilation rate and behavioural profile for *Dascyllus aruanus* envenomated with a medium dose of *Aipysurus laevis* venom.

Figure 6.12: Comparison of ventilation rates and behavioural profile for *Dascyllus aruanus* envenomated with a high dose of *Aipysurus laevis* venom.


Dascyllus aruanus Low Dose
Stages
Ventilation rates / min
Ventilations
Stages
Time in minutes


Dascyllus aruanus Medium Dose
Stages
Ventilation rates / min
Ventilations
Stages
Time in minutes


Dascyllus aruanus High Dose
Stages
Ventilation rates / min
Ventilations
Stages
Time in minutes