EFFECTS OF M.S. 222 ON THE BREATHING AND HEART RATES OF RAINBOW TROUT (Salmo gairdneri)

by

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B. Sc., University of British Columbia, 1968

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

December, 1969

APPROVAL

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Title of Thesis: Effects of M.S. 222 on the Breathing and Heart Rates of Rainbow Trout (Salmo gairdneri)

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ABSTRACT

There are conflicting reports in the literature concerning the effects of anaesthetics on cardiac and respiratory physiology in fishes. The purpose of this study was to investigate the influence of M.S. 222, an anaesthetic widely used in research with teleosts. A total of 96 rainbow trout (Salmo gairdneri) were used. Subcutaneous electrodes for electrocardiogram recording, and buccal or opercular cannulae were inserted while the fish were briefly anaesthetized. After a period of from 2 - 24 hours, the experiments were begun. Heart and respiratory rates were recorded for one hour on a Grass polygraph and a Tektronix oscilloscope. The effects of various concentrations of M.S. 222 (25, 50, 75, and 100 mg/l) were studied at two different temperatures (9° and 17° C). In all cases, except that of 25 mg/l, a gradual decline in both heart and respiratory rates was noticed. Changes in the rates could be correlated to stages of anaesthesia. The respiratory centre was more sensitive to M.S. 222 than the heart and respiratory collapse always preceded cardiac arrest. The E. C. G. pattern remained regular until respiratory collapse occurred. Forcibly ventilating the gills after respiratory collapse, but before cardiac arrest,

immediately normalized the E. C. G. pattern and restored both the heart and respiratory rates to normal within two hours. Although a rise in temperature increases both rates, there appeared to be no significant difference between the effects of the anaesthetic at 9° and 17° C. One of the effects of the treatment was the occurence of synchrony between the heart beat and respiratory movements. At 9° C, the duration of synchrony increased with increasing dosages of the anaesthetic. At 17° C, synchrony was already observed in the control experiment and the anaesthetic did not further increase its duration. Possible regulatory mechanisms (both sensory and in the central nervous system) of cardiac and respiratory functions, and their interrelations, are discussed.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to Dr. P. Belton for allowing me to do this study and for his guidance and patience throughout the preparation of this paper. I want to thank the members of my committee, Drs. K. K. Nair and A. P. van Overbeeke for their helpful suggestions and comments. I am also very grateful to Dr. D. Randall for his assistance whenever called upon and for inciting my interest in fish physiology. Finally, a note of appreciation to all the people in the Department who contributed directly or indirectly to this work.

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INTRODUCTION

Tricaine methanosulphonate, M.S. 222 Sandoz, is widely used as an anaesthetic for fish and is reported to have no apparent after-effects on their physiology (Smith and Bell, 1967). Black and Connor (1964), however, noted slightly lower levels of lactate in muscle and blood of fish treated with M.S. 222. They concluded that the difference was due to a difference in sampling time and the absence of struggling during the sampling in the anaesthetized animals. Randall (1962) observed a significant increase in the heart rate and variable increases in the respiratory rate and amplitude in the tench, Tinca tinca L., with low doses of M.S. 222, whereas larger doses caused cardiac irregularities and respiration ceased. These effects were reversible. Serfaty et al. (1959) examined the effects of M.S. 222 on the heart of the carp. McFarland (1959) studied the effects of anaesthetics on certain aspects of the physiology and behaviour in Fundulus, Girella, and Paralabrax. Campbell and Davies (1963) investigated the anaesthetic effects of M.S. 222 in elasmobranchs. The findings of all these workers indicate that M.S. 222 does not produce consistent effects with respect to heart and

breathing in fish. There is no relevant study on the effects of M.S. 222 on respiratory and cardiac rhythms of the rainbow trout, Salmo gairdneri.

Anaesthesia is a physiological state produced by many agents in which the normal responsiveness of the nervous tissues and excitable cells is temporarily decreased or abolished. The degree to which sensitivity is decreased is variable and may range from a slight sluggishness to a complete loss of response. An essential characteristic of anaesthesia is its reversibility or the ability of the tissues to return to normal functioning upon removal of the anaesthetizing agent. This reversibility distinguishes anaesthesia from death where the loss of response is irreversible. In a study of the properties of various anaesthetics, McFarland (1959) has distinguished the following stages of anaesthesia in fish:

Stage 0: This is defined as normal behaviour in the absence of anaesthetics.

Stage 1: It is characterized by a partial loss of responsiveness to external stimuli.

Plane 1: In this phase of stage 1, there is reduction in the fright response to visual stimuli. The fish move more slowly when stimulated and sink to the

bottom very slowly. Equilibrium is maintained and a slight dispersion of melanin may occur. The fish are less active but have a normal breathing rate.

Plane 2: There is a lack of response to external stimuli but the equilibrium is still undisturbed. The fish show no sign of distress. During this stage the pectoral and caudal fins move in a manner compensatory to the respiratory currents, i.e. the coordination of swimming with respiration may be advantageous, ensuring that the mouth opens as the thrust of the trunk and tail force the fish forward into the water.

Stage 2: In this stage there is a partial to total loss of equilibrium. The fish become darker and the opercular rate becomes abnormal.

Phase 1: Swimming becomes uncoordinated and there is loss of equilibrium. The fish darken and the opercular rate increases.

Phase 2: There is complete loss of equilibrium and cessation of swimming movements. The fish lie upside down on the bottom of tank. They do not react to stimuli even if they are lifted from the water. The

opercular rate declines rapidly.

Stage 3: There is severe disturbance of the respiratory rate. Equilibrium is completely lost and the fish become very dark.

Stage 4: Respiratory and cardiac collapse occur. Respiratory movements cease, the opercules are spread and pectoral fins are usually in the extended position. The fish lie upside down in the tank. No recovery is possible if the fish are left in the anaesthetic solution but if they are removed and placed in fresh water the conditions can be reversed and the functions return to normal. McFarland (1959) considers the fish dead when opercular movements have ceased for one minute even though slow contractions continue in the heart for several minutes after cessation of the opercular movements.

Ventilation of the respiratory surfaces of vertebrates is the result of coordinated contractions of skeletal muscles termed breathing. These contractions are normally rhythmic and under the control of a group of neurons in the medulla oblongata collectively referred to as the medullary respiratory center. Similar rhythmic movements are often seen in other

groups of skeletal muscles. All skeletal muscle activity is directly controlled by the nervous system, and neuronal systems controlling skeletal activity must be arranged in such a way as to permit coordinated rhythmic patterns like walking, running or swimming.

Patterns such as ventilation are normally involuntary functions whereas running, walking or swimming are usually voluntary activities. Under certain conditions however, such as extreme stress (Randall and Shelton, 1963), strenuous exercise (Falls, 1968), and anaesthesia (von Holst, 1934a, b) these voluntary patterns become synchronized with the breathing and heart movements. Von Holst (1934a, b) observed synchronized movements of the lateral dorsal musculature and the breathing muscles of fish under anaesthesia. Randall (1967) noted synchrony of breathing with sinus arrythmia in fish. Schoenlein (1895) reported synchrony between heart beat and respiration in various fish under normal conditions; however, Baglioni (1906) was unable to substantiate these results. Synchrony was also observed by Babak (1912), Lyon (1926), Lutz (1930a, b), and Satchell (1961). Willem (1921) suggested that the cardiac rhythm regulates the respiratory rhythm. Labat (1966) has also suggested a close association between

cardiac and respiratory centers with the heart playing the major role in regulation. Von Holst (1934a, b) postulated the existence of a single automatic rhythmical process in the anterior region of the spinal cord whose activity represents the common basis for respiratory, pectoral fin and tail movements. He observed that the various rhythms became synchronized under specific levels of anaesthesia. In his opinion, the common generator to all rhythmic movements would be subject to the sensory input of each element under its control. Exteroceptive or proprioceptive stimulation would then result in a variation of individual excitation and a consequent asynchrony of the three movements. Von Holst emphasized that the fundamental rhythm itself is an automatic process independent of the periphery. Shelton (1961) has described the respiratory center of teleosts as being situated in the medulla oblongata. Therefore, if a single oscillator controlling these movements exists it should also be located in the medulla. This also seems to be supported by the work of von Holst. If he placed a goldfish in a weak solution of urethane, he was able to demonstrate a close relationship between the various movements under consideration. Further, he was able to demonstrate that synchrony occurred when the region anterior to the medulla oblongata was almost or completely out of action, i.e. when the reception from the

periphery was greatly reduced or stopped. He considered the stopping of impulses from the periphery to be essential since unanaesthetized animals with a cut in front of the medulla did not show synchronization. If we are to accept the idea of an oscillator regulating the three movements mentioned by von Holst, the simplest explanation would be that breathing is regulated by an oscillator situated in the respiratory center and that the other functions are controlled by this oscillator in the absence of sensory input. A simple numerical relationship between heart and breathing rates has been known for a long time, yet it was not until 1960 that Satchell demonstrated in the dogfish that the heart beat occurred at a very specific time in the breathing cycle so as to make maximum water flow over the gills coincide with the maximum blood flow through the gills. Serfaty and Raynaud (1957) recorded the respiratory movements and electrocardiograms from teleosts and found that synchronization between heart beat and breathing was rare and persisted for very short times only. Hughes (1961) claimed to have observed occasional synchronization in the trout with the heart beating at a specific point in the respiratory cycle. Randall (1962) reported that a trout lightly anaesthetized with M.S. 222 showed a marked tendency for the heart beat and

breathing to become synchronized in a 1:1 ratio, although it could occur in some multiple of this. He could not show any strict correlation between the timing of the heart beat and any part of the respiratory cycle but there was a marked tendency for the heart to beat during the mouth closing phase of the cycle. The concentration of M.S. 222 required to produce this synchrony could not be predicted nor could the synchrony be produced with any degree of certainty on any given occasion. He did make the suggestion that some connection might exist between cardiac and respiratory centers within the brain. As early as 1842, Flourens demonstrated that the rhythmical activity of the respiratory center was completely independent of regions anterior and posterior to the medulla oblongata. Adrian and Buytendijk (1931) were the first to record changes in electrical potential associated with activity of the respiratory center in fishes. They used the isolated brain of the goldfish Carassius auratus with the forebrain removed. Because this isolated preparation was not receiving afferent stimuli, it was concluded that these potentials were the result of spontaneous automatic excitations within the respiratory center itself.

One of the most critical considerations biologists must bear in mind is the extent to which their techniques affect

their results. Control experiments in which normal functioning can be recorded are obviously very difficult to design and not all experimentation can be carried out on unrestrained unanaesthetized animals. The purpose of this study was two-fold. Firstly I examined some of the changes occurring in heart rates and respiratory rates when rainbow trout are being handled, are exposed to different temperatures and are under the influence of anaesthetics. The anaesthetic used was M.S. 222 Sandoz (Tricaine methanosulphonate), because even though this drug may not have any obvious after-effects, it is very likely that the anaesthetic produces various effects during its active period and these effects should not be overlooked when the experimenter is interpreting the results obtained. Secondly, an attempt was made to analyze the phenomenon of synchrony observed between breathing and heart movements under anaesthesia.

MATERIALS AND METHODS

1. Fish and general maintenance procedures.

The experiments were carried out on ninety six rainbow trout (Salmo gairdneri) weighing approximately 250 grams. The fish were purchased from the Sun Valley Trout Farm, Mission, B. C. In the laboratory, the fish were held in large, 175 gallon, holding tanks supplied with a continuous flow of dechlorinated water, for approximately four weeks prior to any experimentation. This was done to allow the fish to become adapted to their new environment. The temperature of the water varied from a low of 6° C in the winter to a high of 11°C in the summer. The fish were fed every second day with a commercial pellet-type fish food purchased from the trout farm. During the experiments fish were taken from the holding tanks and placed directly in a solution of Tricaine methanosulphonate (M.S. 222 Sandoz). They remained in this solution until anaesthesia had set in to the point of loss of balance, spontaneous movements and response to external stimuli, i.e. Stage 2 - Phase 2 (McFarland).

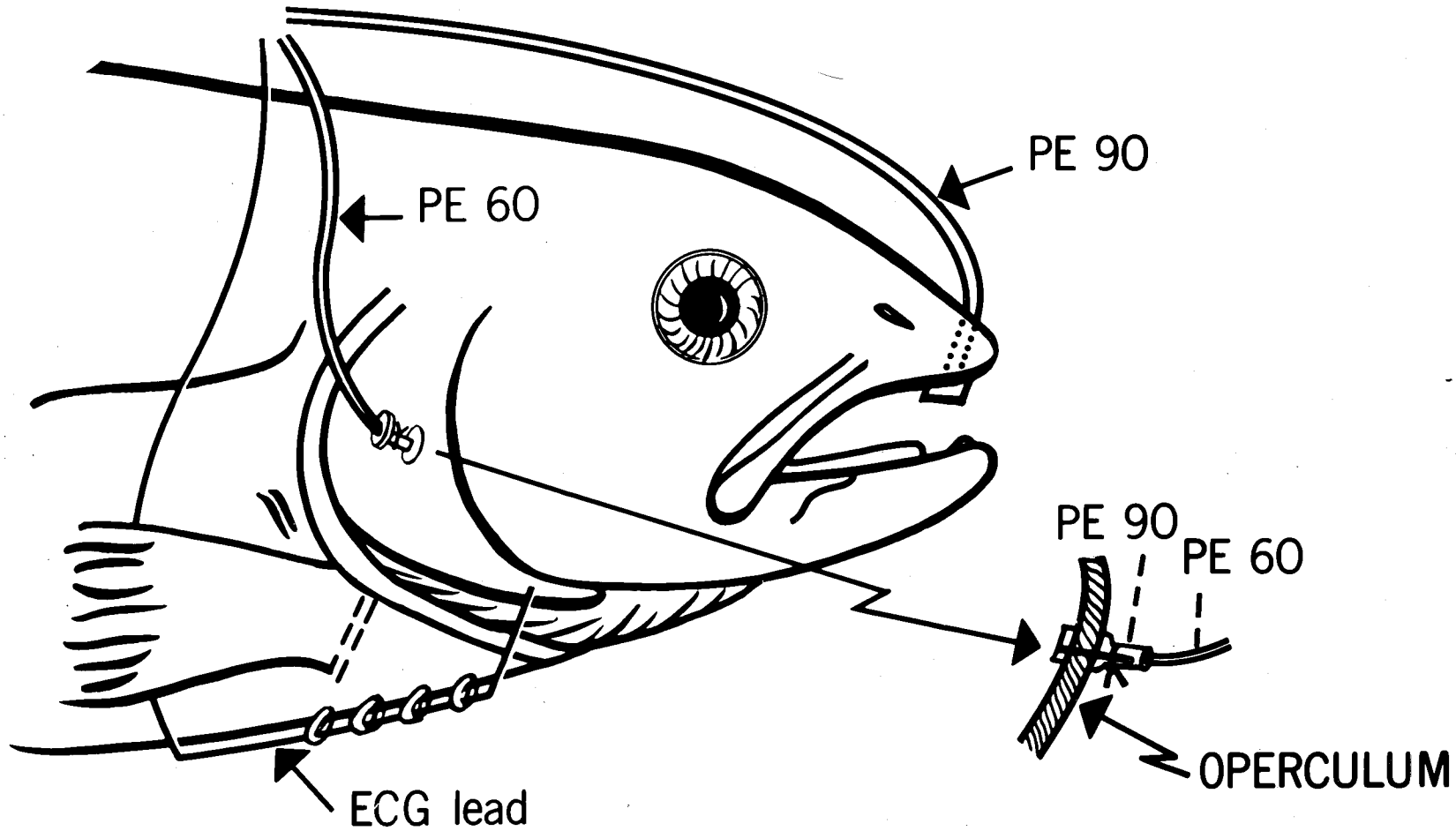
2. Recordings of heart and respiratory rate.

While under anaesthesia, the fish were cannulated either

Figure 1: Details of the head of the trout showing relative positions of cannulae and electrode.

PE 60)
) represent cannulae used for recording pressure
PE 90) changes.

E. C. G.: electrode for recording electrocardiogram.


PE 90
PE 60
PE 90
PE 60
OPERCULUM
ECG lead

in the buccal or in the opercular cavities to determine breathing rates by recording pressure differences in the fluid system. The technique used for inserting the cannulae was modified from that described by Saunders (1961). The following procedures were carried out (see Figure 1):

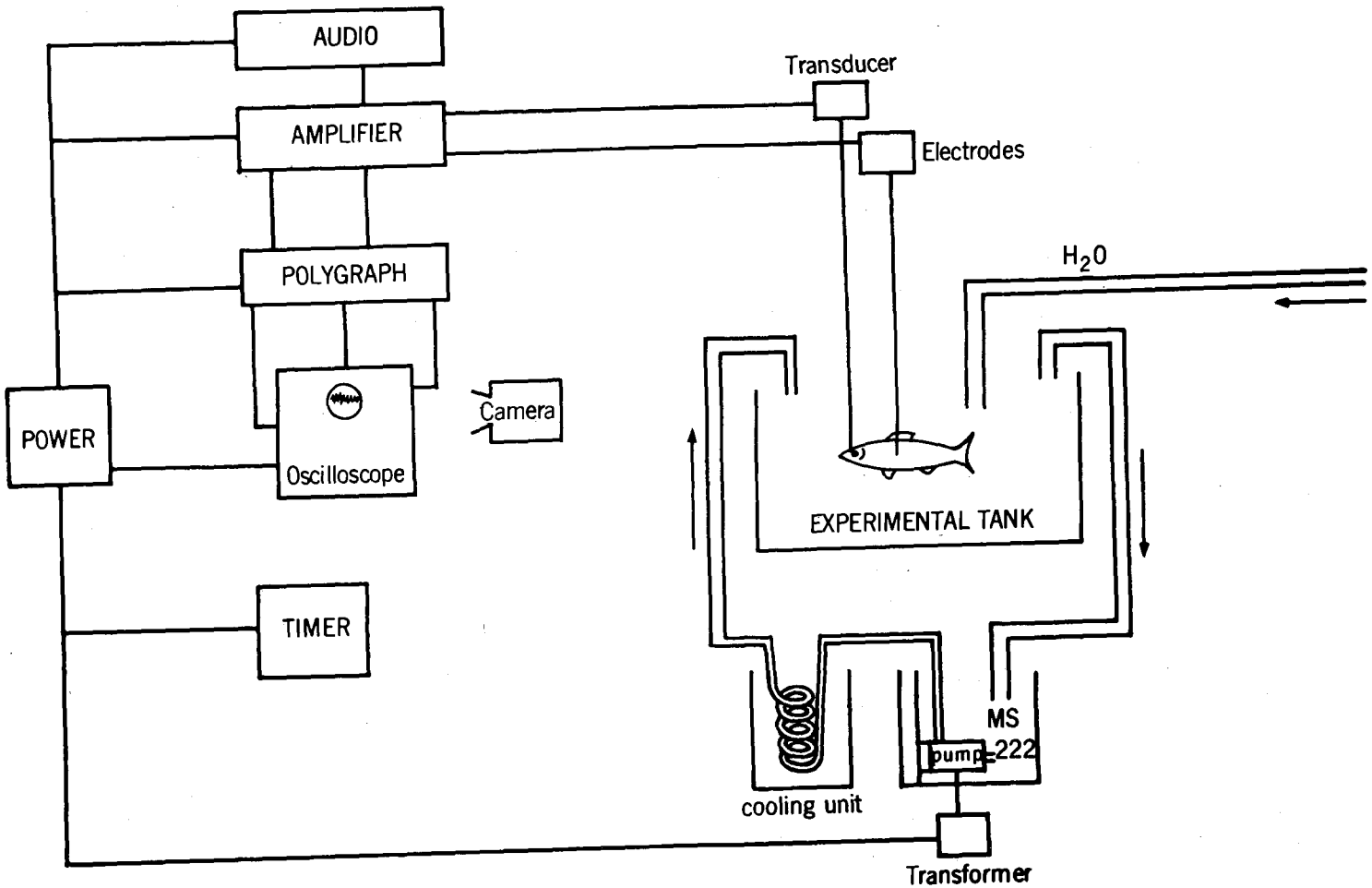
a) Buccal cavity cannulation: a hole was punched in the cartilaginous portion of the snout in the dorsal mid-line using a 14 gauge hypodermic needle. Care was taken to avoid damage to the oral valves and the olfactory lobes. Eighty centimeters of PE 90 polyethylene tubing (Clay-Adams) was passed through the hole in the snout. The end of the tubing was heat flared to anchor it. Thread was wrapped around the tubing where it emerged from the snout to prevent inward movement of the cannulae.

b) Opercular cavity cannulation: the cavity was cannulated by punching a hole in the center of the operculum with an 18 gauge hypodermic needle and anchoring a heat-flared eighty centimeter length of PE 60 polyethylene tubing (Clay-Adams) in the hole (Saunders, 1961). One centimeter of heat-flared PE 90 tubing was passed over the outside of the opercular cannula. This short piece of tubing was used in this application to maintain the cannula snug against the operculum. The whole system was

secured with a piece of thread. PE 60 tubing was used in order to maintain a high degree of flexibility so as not to impair the normal function of the operculum. Care was taken that all cannulae were free of constrictions and of air bubbles. Although the fish were not restrained per se it was necessary to maintain the cannulae short enough to prevent the fish from becoming entangled. Eighty centimeters was adequate for this purpose. An advantage of the shorter cannula was to reduce the loss of response of the system.

The cannulae were then connected to a Statham P 23 AA pressure transducer, the output of which was displayed on a Grass Polygraph recorder (Figure 2). The electrocardiogram was taken by inserting an E 2B Platinum needle subcutaneous electrode (Grass) in the ventral mid-line just cephalad to the pectoral fins. The reference electrode was inserted in the myotomes caudad to the dorsal fin. The E. C. G. was also displayed on the polygraph. An output lead from each channel of the polygraph was then connected to a 565 Tektronix oscilloscope where both signals were added using a Dual-Trace Amplifier and played back on a third channel of the polygraph. This enabled me to simultaneously record the heart beat and respiration. Throughout the experiment the fish were relatively

Figure 2: Diagram of the arrangement of the experimental equipment.


AUDIO
AMPLIFIER
POLYGRAPH
POWER
Oscilloscope
Camera
TIMER
Transducer
Electrodes
H_2O
EXPERIMENTAL TANK
MS
222
pump
cooling unit
Transformer

unrestrained in a small tank of 45 liters capacity through which a continuous flow of either fresh water or experimental solution could be maintained. During the course of the experiments, except for the temperature experiments, the temperature did not vary more than - 0.5° C. After inserting the cannulae the fish were left undisturbed in the small tank for periods of two to twenty four hours to allow heart rate and respiratory rate to attain steady values which were then considered to be the resting values. The experimental periods were of 60 minutes duration unless the fish went into respiratory collapse. In this case they were revived by artificially ventilating the gills with fresh water. At no time were the fish allowed to complete Stage 4 (McFarland) and die.

To study the effects of the anaesthetic, four concentrations of M.S. 222 were used, namely: 25, 50, 75, and 100 mg/l.

To study the effects of temperature, heart and respiratory rates were recorded from fish which were subjected to a gradual increase in temperature from 9° to 27° C during a period of 60 minutes. Recordings from each fish in the experimental conditions were made for one hour: every minute for the first ten minutes, every 2½ minutes for the next twenty minutes, and every 5 minutes thereafter until the 60 minute limit was reached.

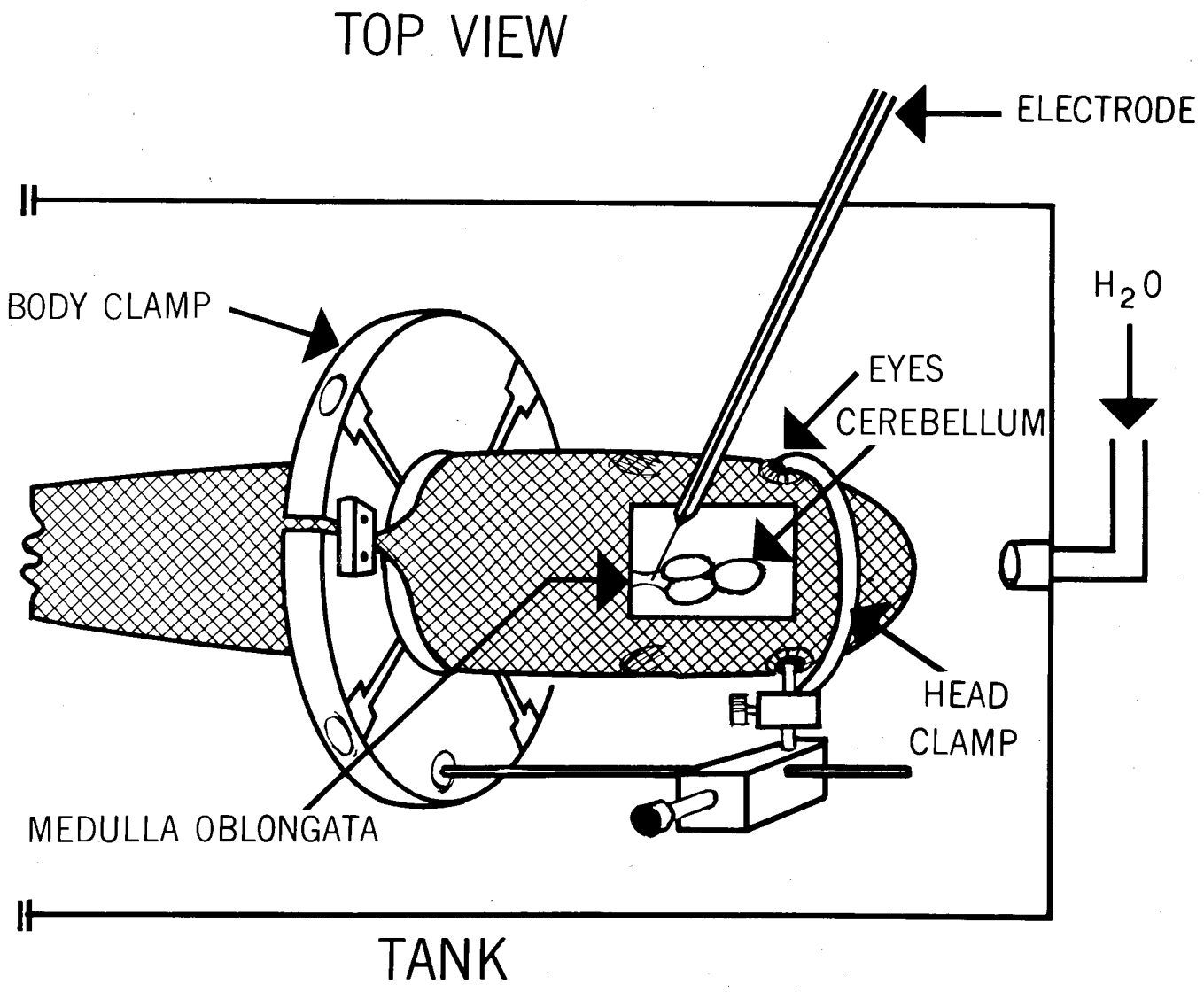
3. Electroencephalographic recordings.

Recordings from the medulla oblongata were obtained by placing the fish in a clamp mounted in a box of 5 liters capacity. An anaesthetic solution was passed through the box continually. A metal clamp attached to the body clamp fitted across the supraorbital ridges of the fish (Figure 3). The brain was exposed by cutting through the cartilaginous skull with a dentist's drill, taking care not to injure any brain tissue. The incision was made from a line vertical to the eye to a line just posterior to the operculum, in the mid-dorsal part of the snout. The electroencephalogram was recorded with a 0.003 mm teflon coated platinum alloy electrode. The output was displayed on a 565 Tektronic oscilloscope. The oscilloscope was set to filter out low frequencies resulting from contractions of skeletal and cardiac muscle. All leads were made of a double-shielded wire. Selected recordings were photographed with a Polaroid camera.

4. Evaluation of methods.

The immediate survival of inserting the cannulae was 100%. However, the length of survival in the experimental tank was variable and therefore it was very difficult to attribute death to any particular cause. A tissue reaction to the cannulae was

Figure 3: Diagram showing the clamps holding the head of a trout and the exposed brain, after preparation for E. E. G. recording.


TOP VIEW
ELECTRODE
BODY CLAMP
H_2O
EYES
CEREBELLUM
HEAD
CLAMP
MEDULLA OBLONGATA
TANK

noted after approximately 7 days. A general decrease in activity was also noted in these fish after about 10 days. Since the experiments were performed within 48 hours of the insertion of the cannulae, these factors did not in all likelihood affect the results.

RESULTS

I. Electrocardiograms

The typical E. C. G. recording of trout consists of the P, QRS, and T waves (Figure 4). The atrio-ventricular time difference as measured by the difference between the P wave and QRS complex ranged from 0.15 to 0.35 seconds with a mean value of 0.20 seconds. The general shape of the E. C. G. of fish is similar to that of most vertebrates. It consists of an initial rounded deflection, the P wave, resulting from the passage of excitation through the atrial muscle; a second deflection, the QRS complex, which is a biphasic complex produced by the activation of the ventricle; and a final deflection, the T wave, resulting from repolarization of the ventricle.

Electrocardiograms have been recorded for a number of fish, including the tench, carp and eel (Oets, 1950) and elasmobranchs and salmonids (Kisch, 1948). Oets found, in the eel, in addition to the waves normally seen, a V wave which was slightly superimposed upon the P wave and resulted from the activity of the sinus venosus. Kisch (1948) reported a B wave in Selachians between the QRS complex and the T wave, corresponding to the

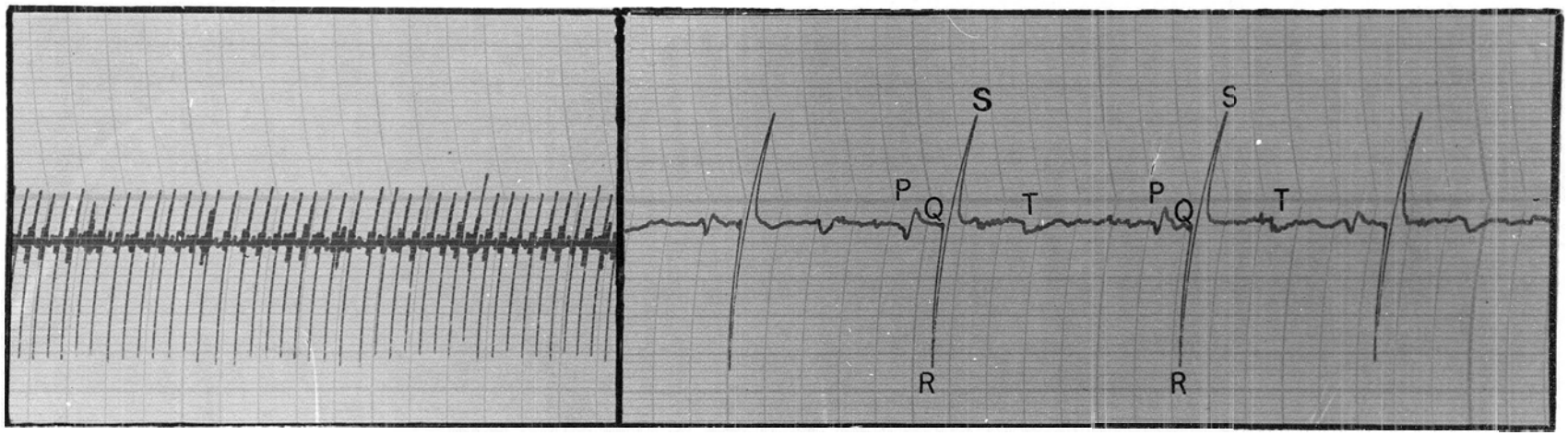
Figure 4: Typical electrocardiogram recorded from a rainbow trout.

Temperature 9° C.

Chart Speed: a) 2.5 mm/sec

b) 10 mm/sec

P, QRS, T represent the waves of excitation passing over the heart.


S
S
P
Q
T
P
Q
T
R
R

activation of the bulbis cordis.

No V wave or B wave were noted in my experiments. This would seem to indicate that in salmonids, the bulbus and sinus are non-contractile; or that the B wave overlaps the T wave and the V wave overlaps the P wave.

a) Effect of handling:

Handling in this experiment refers to the procedure of inserting the cannulae or the transfer of the cannulated fish from one tank to another. Handling as transferring the fish caused a decrease in the heart rate. This response is immediate (Figure 5). Some asystole was observed during this initial response but after about ten minutes the heart beat became regular again. A stable rate was reached within one to two hours and these values were then considered to be the resting rates for that fish. Once the heart rate had regained its regularity no further changes were observed in either amplitude or patterns of the E. C. G. complex.

b) Effect of temperature:

Raising the water temperature had drastic effects on the heart rate of the trout (Figure 6). An increase of the temperature from 9 to 27° C is accompanied by an increase in the heart rate of 284% of its resting value. This increase is

Figure 5: Effect of handling rainbow trout on the heart rate and respiratory rate.

Temperature 9° C.

o—o Respiratory Rate

●—● Cardiac Rate

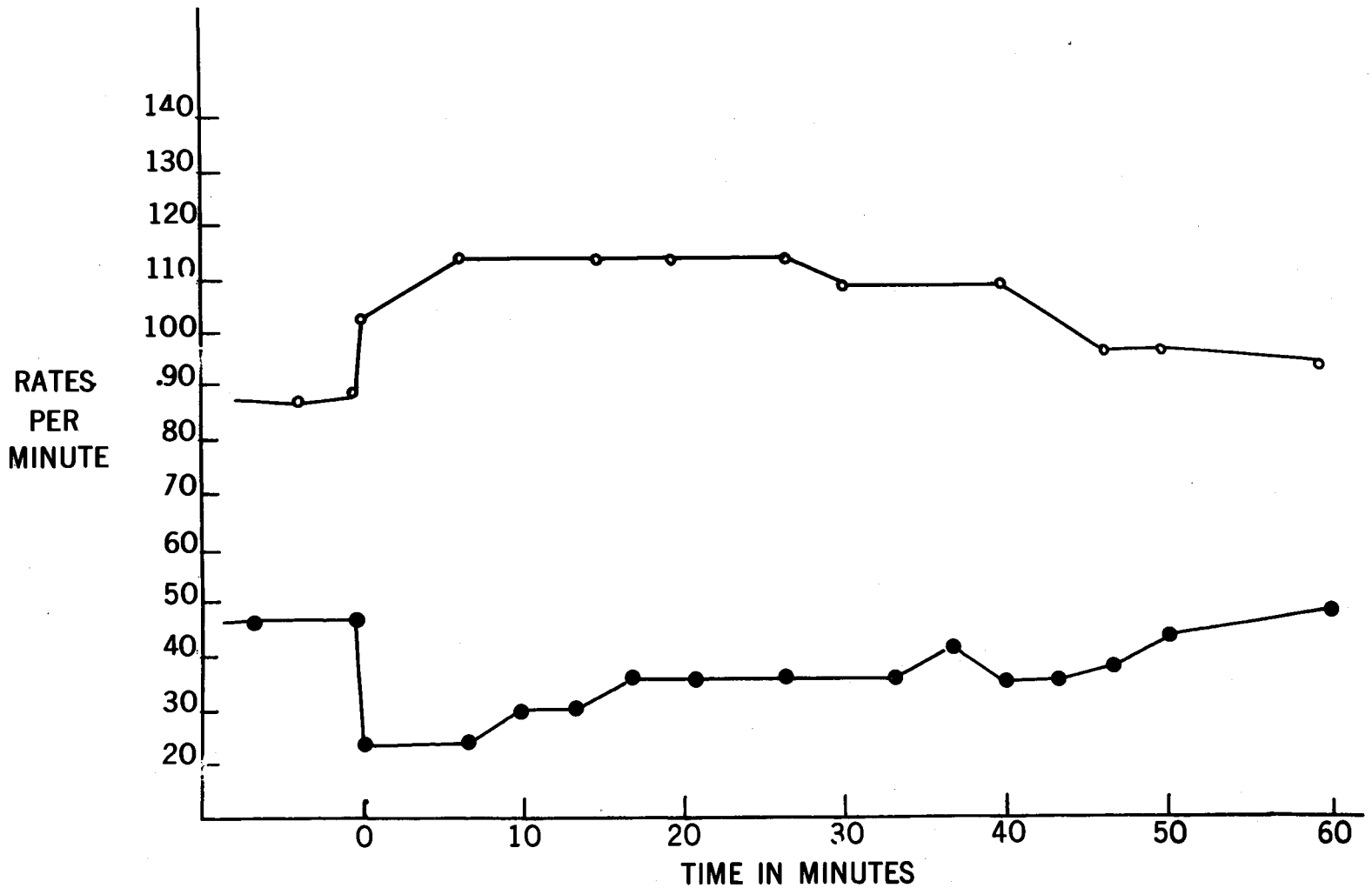
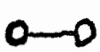

RATES PER MINUTE
140
130
120
110
100
90
80
70
60
50
40
30
20
0
10
20
30
40
50
60
TIME IN MINUTES

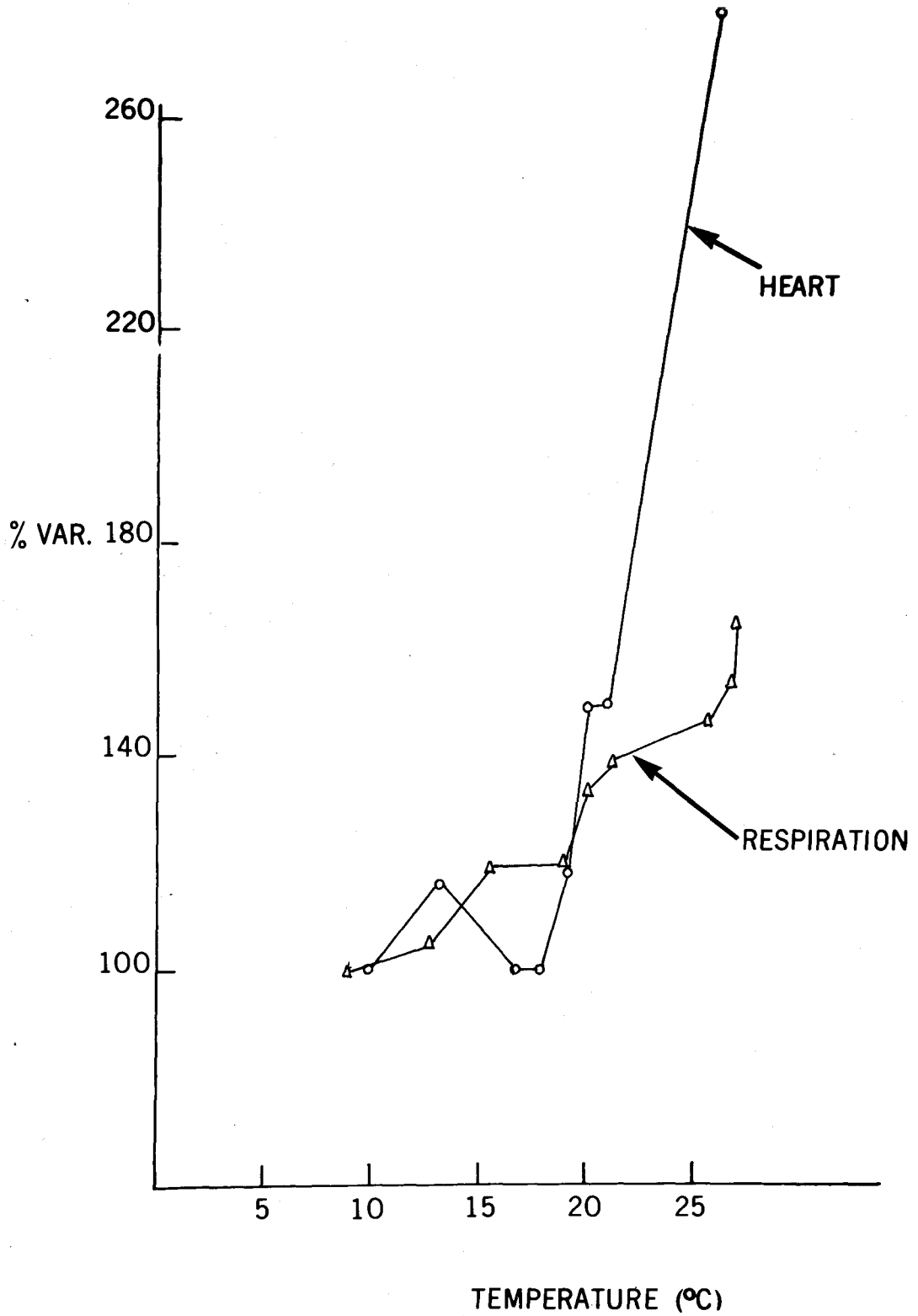
Figure 6: Effect of increasing water temperature on the cardiac and respiratory rates expressed as percentage of the resting values of the rainbow trout. Resting values are considered to be 100%.


Heart Rate


Respiratory Rate

Duration of experiments: 60 minutes.

Number of fish: 6.


260
220
% VAR. 180
140
100
HEART
RESPIRATION
5
10
15
20
25
TEMPERATURE (°C)

not linear however. The heart rate remains fairly stable until 19° C and at 20° C the amplitude of the QRS complex becomes much larger probably indicating a stronger cardiac stroke which would result in a larger cardiac output. At temperatures above 20° metabolic functions are so heavily taxed that eventually they collapse. This is referred to as the zone of resistance (Hoar, 1966). No trout are found at temperatures above 20° C in nature. The cardiac rate increased from 140 to 284% of its resting value for a change in temperature from 19 to 27° C. Because of these results, 17° C was used in later experiments to study the effects of anaesthetics at higher temperatures.

c) Effect of M.S. 222:

Following the introduction of Sandoz M.S. 222 in the medium there are very definite changes in the heart rate (Figures 7 and 8).

Corresponding to Stage 1 of anaesthesia, there was an increase in the heart rate at all concentrations and at both temperatures (Figures 7 and 8). The results for 50 mg/l at 9° C showed an initial increase of only 2%. This increase was followed by a decrease which denotes the transition from Stage 1 of anaesthesia to Stage 2. At a concentration of 25 mg/l

Figure 7: Effect of various concentrations of M.S. 222 on the heart rate expressed as percentage of the resting values of the rainbow trout. Resting values are considered to be 100%.

Temperature 9° C.

Number of fish in each of the four groups: 10.

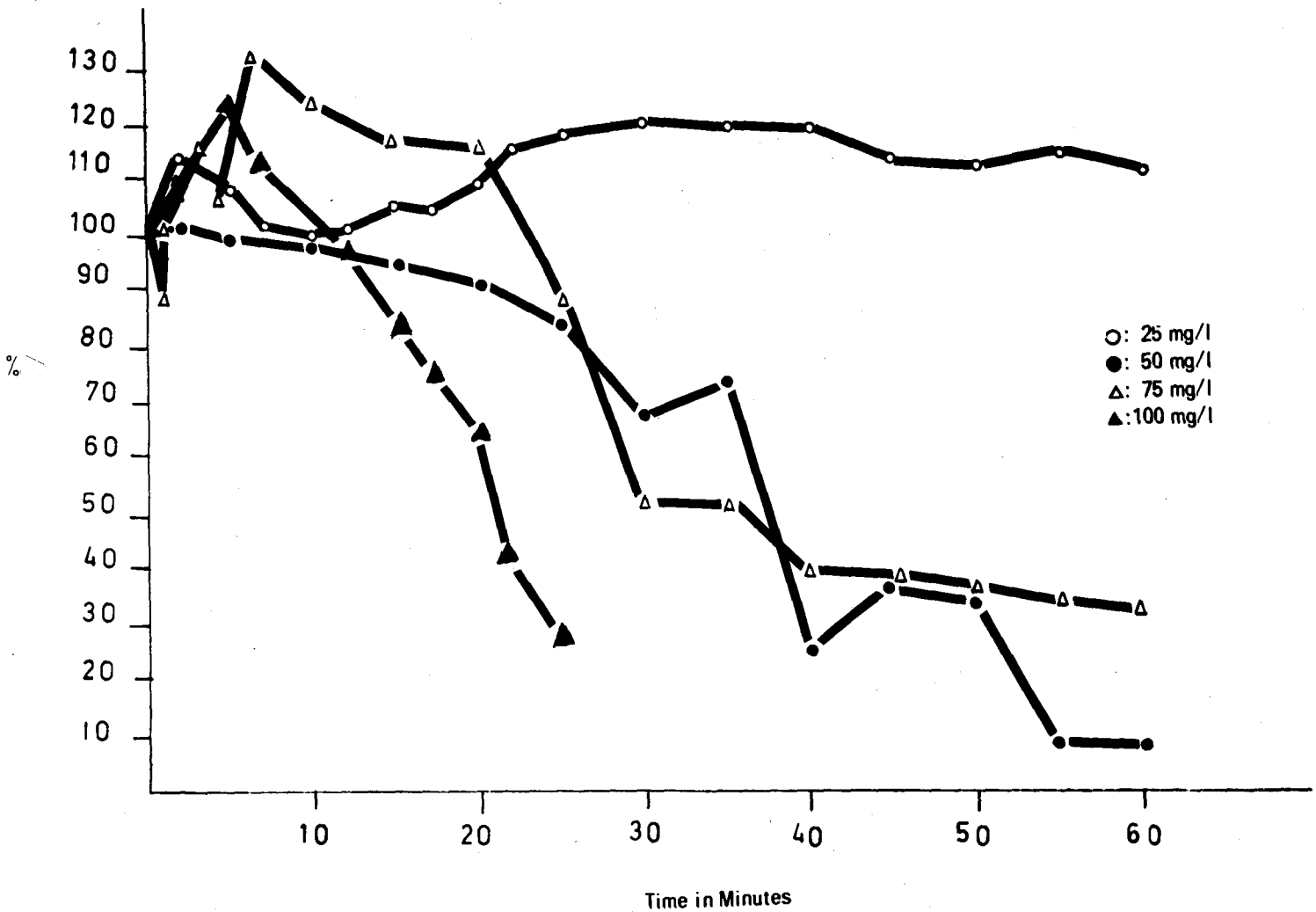
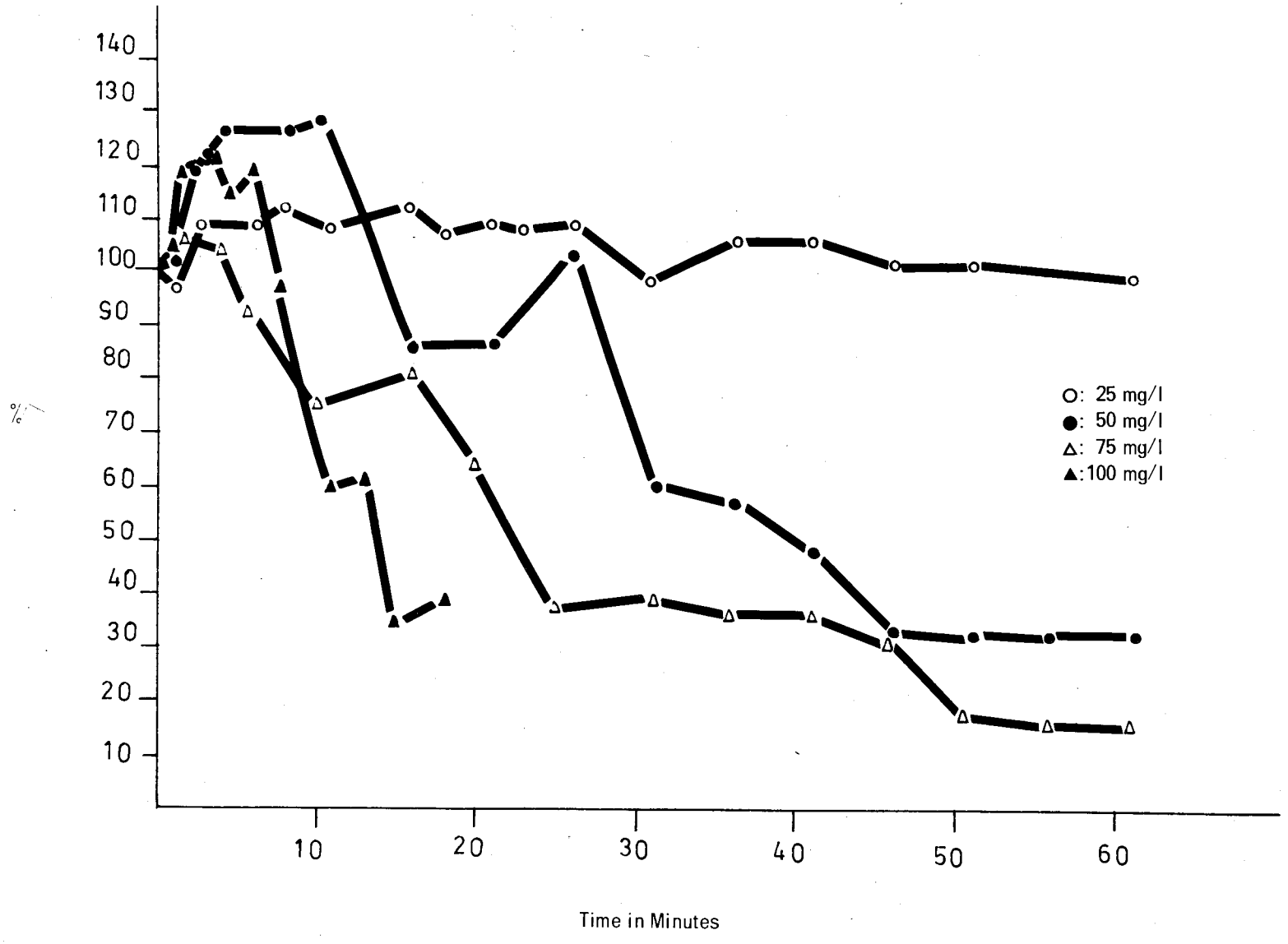

130
120
110
100
90
80
70
60
50
40
30
20
10
%
10
20
30
40
50
60
Time in Minutes
○: 25 mg/l
●: 50 mg/l
△: 75 mg/l
▲:100 mg/l

Figure 8: Effect of various concentrations of the anaesthetic on the heart rate expressed as percentage of the resting values of the rainbow trout. Resting values are considered to be 100%.

Temperature 17° C.

Number of fish in each of the four groups: 10.


140
130
120
110
100
90
80
70
60
50
40
30
20
10
%
10
20
30
40
50
60
Time in Minutes
O: 25 mg/l
●: 50 mg/l
△: 75 mg/l
▲:100 mg/l

(9 and 17° C) the heart rate stabilized within ten minutes at a rate somewhat higher than the resting value. At the other concentrations the rate continued to decline until respiratory collapse (Stage 3). Within minutes after the onset of Stage 3 cardiac collapse also occurred. Although the tendency was for the heart rate to decline with time after the initial increase, the heart beat (composition of E. C. G.) remained normal until respiratory collapse at which time some asystole and changes in the QRS complex are observed (Figure 9).

During the initial period there is no change in the distance between the various waves of the electrocardiogram which suggest that M.S. 222 does not appear to affect the conduction rates of the waves of excitation. The changes occurring later were probably the result of the hypoxic conditions created by respiratory collapse. A new slow rate persisted until cardiac arrest. This new rhythm seems to be due to the myogenic nature of the vertebrate heart and probably appears after the nerves controlling the heart are blocked by the anaesthetic in Stage 3. As long as this pacemaker potential persisted, the fish could be revived by forcibly ventilating the gills at both temperatures. If this was done the heart beat returned to values approaching the resting values within 0.5-1 second

Figure 9: Recordings of the electrocardiogram:

A) Normal recording

B) Changes in E. C. G. complex after respiratory collapse and onset of a new rhythm.

Temperature: 17° C.

Concentration of M.S. 222: 100 mg/l.

Chart Speed: 2.5 mm/sec.

Upper Trace: timer.

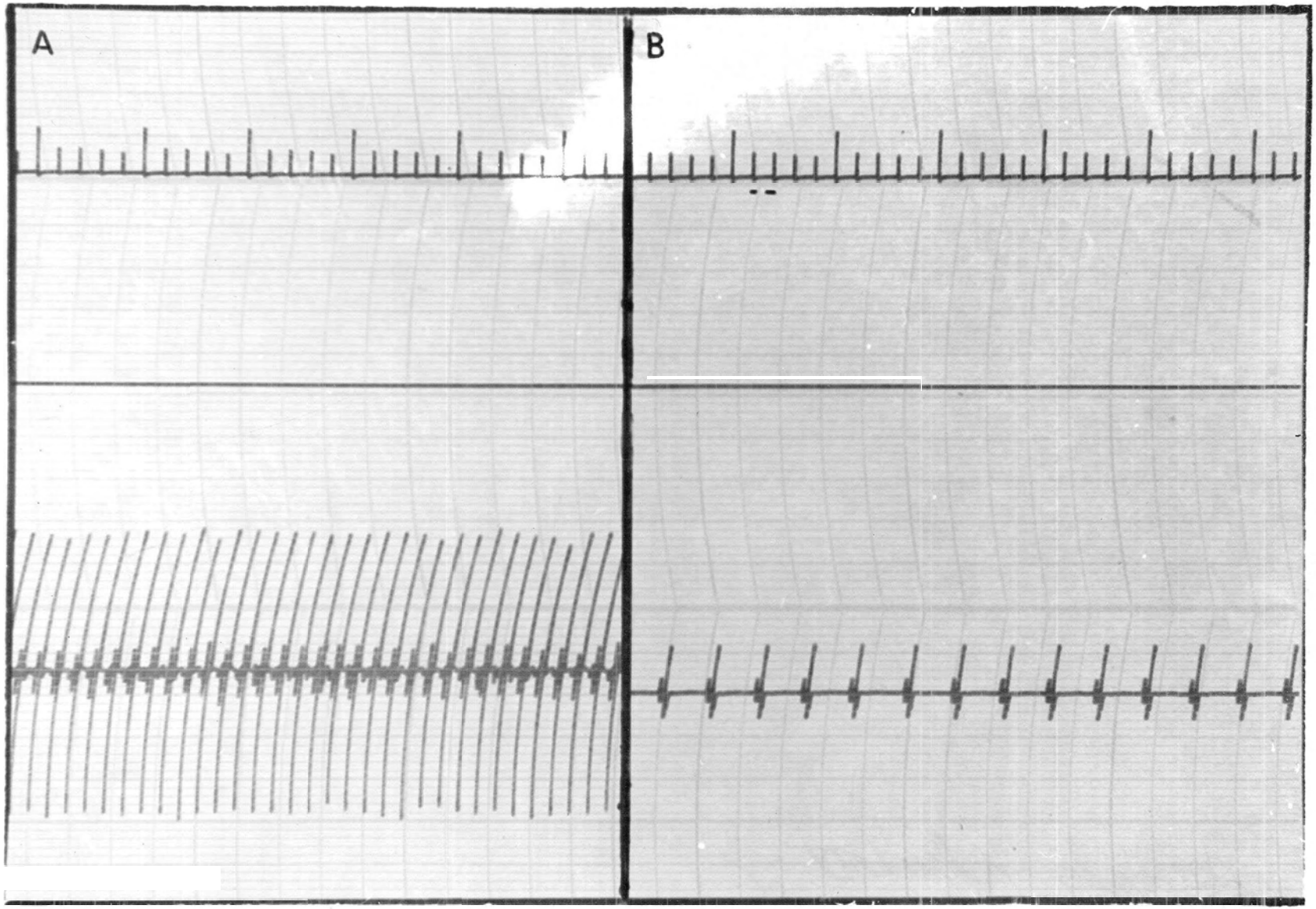
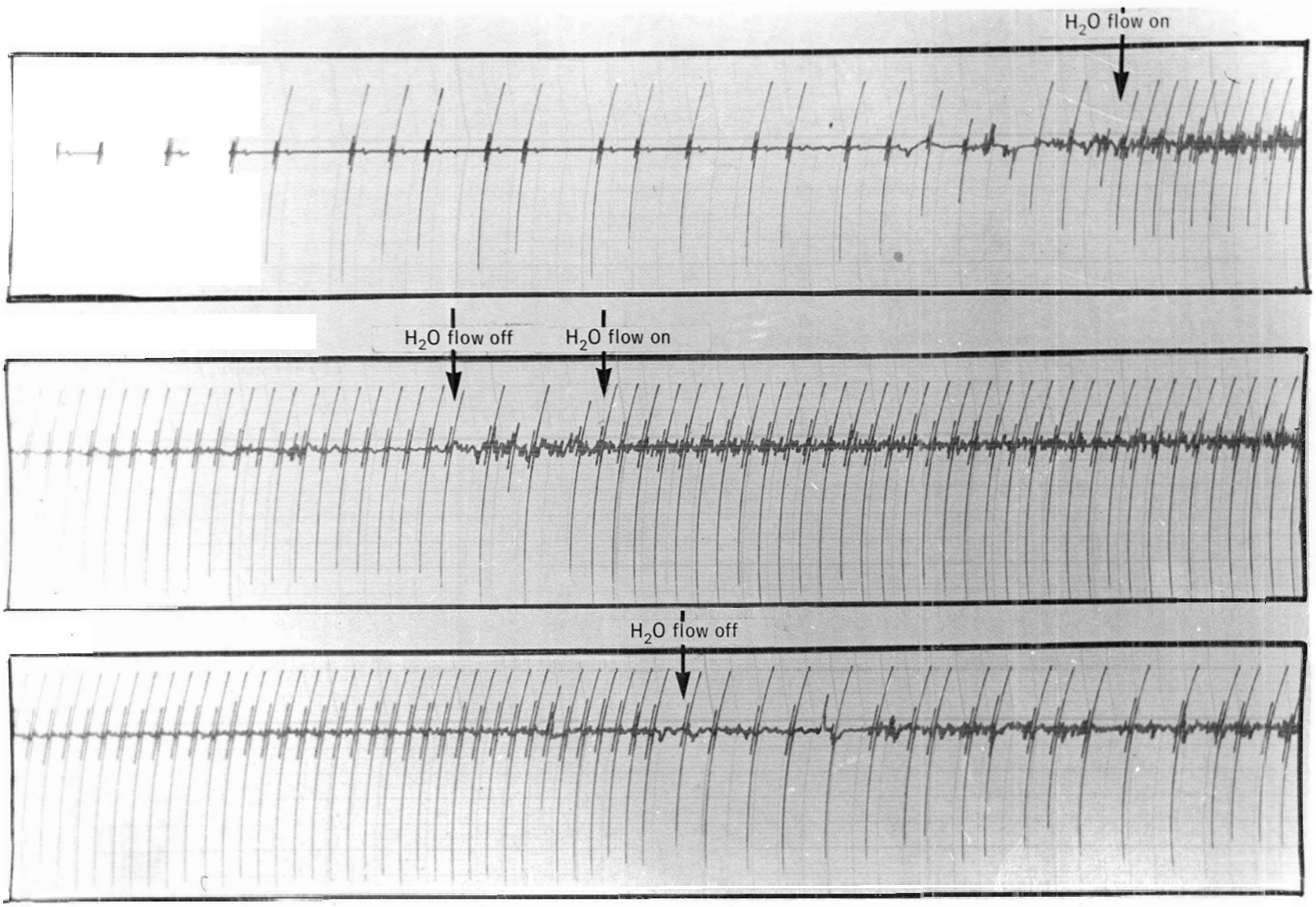

A
B

Figure 10: Effect on the heart rate of the rainbow trout of forcibly ventilating the gills after respiratory collapse.

Temperature: 9° C.

Concentration of M.S. 222: 100 mg/l.


H_2O flow on
H_2O flow off
H_2O flow on
H_2O flow off

(Figure 10).

There was no significant difference between the effects of the anaesthetic on the heart rate in the same concentrations at 9 and 17° C (Figures 11 - 14, Appendix 1 - 4). However, at 17°C, darkening and loss of equilibrium developed more rapidly. Also, at this last temperature respiratory collapse occurred more frequently (Table I, Page 31) and sooner. The time of recovery is increased not only by increasing the concentration of the anaesthetic but also by raising the temperature. It should be noted that the initial resting values are significantly different at the two temperatures but the percentage variations from these resting values do not appear to be significant.

II. Respiratory Cycle

The mechanism of gill ventilation has been well described by Hughes and Shelton (1962). Water flow over the respiratory epithelium is maintained more or less continuously by means of a double pump mechanism. There does however, appear to be a period of maximum water passage over the gills in teleosts during the "mouth closing" phase of the respiratory cycle. As the mouth closes, increases in pressure in the buccal cavity rise very sharply (Figure 15) and it reaches a maximum when the mouth is almost completely closed (in other words, when the

TABLE I

Percentage of fish undergoing respiratory collapse in various concentrations of M.S. 222 at 9° and 17° C.

CONCENTRATION OF M.S. 222	PERCENTAGE at 9° C	at 17° C
25 mg/l	0	0
50 mg/l	30	60
75 mg/l	70	100
100 mg/l	100	100

Total number of fish: 80

Figure 11: Effect of 25 mg/l M.S. 222 on the heart rate expressed as percentage of the resting values of rainbow trout at 9° and 17° C. Resting values are considered to be 100%.

Number of fish for each of the two groups: 10.

The values at the extreme right were taken 60 minutes after the end of the experiment.

Bars: Represent Standard Errors

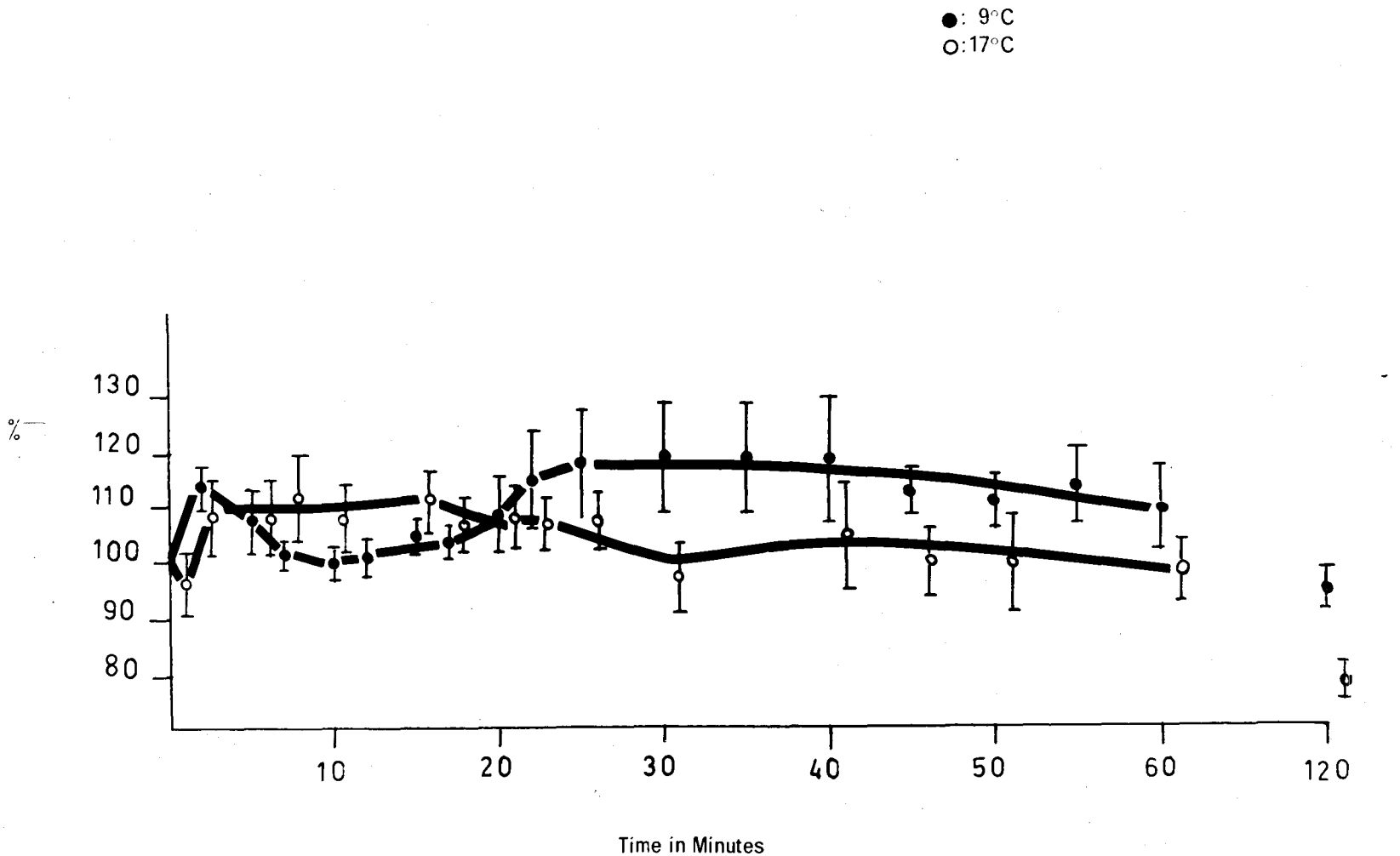

●: 9°C
○:17°C
%
130
120
110
100
90
80
10
20
30
40
50
60
120
Time in Minutes

Figure 12: Effect of 50 mg/l M.S. 222 on the heart rate expressed as percentage of the resting values of rainbow trout at 9° and 17° C. Resting values are considered to be 100%.

Number of fish for each of the two groups: 10.

Values at the extreme right were taken 60 minutes after the end of the experiment.

Bars: Represent Standard Errors

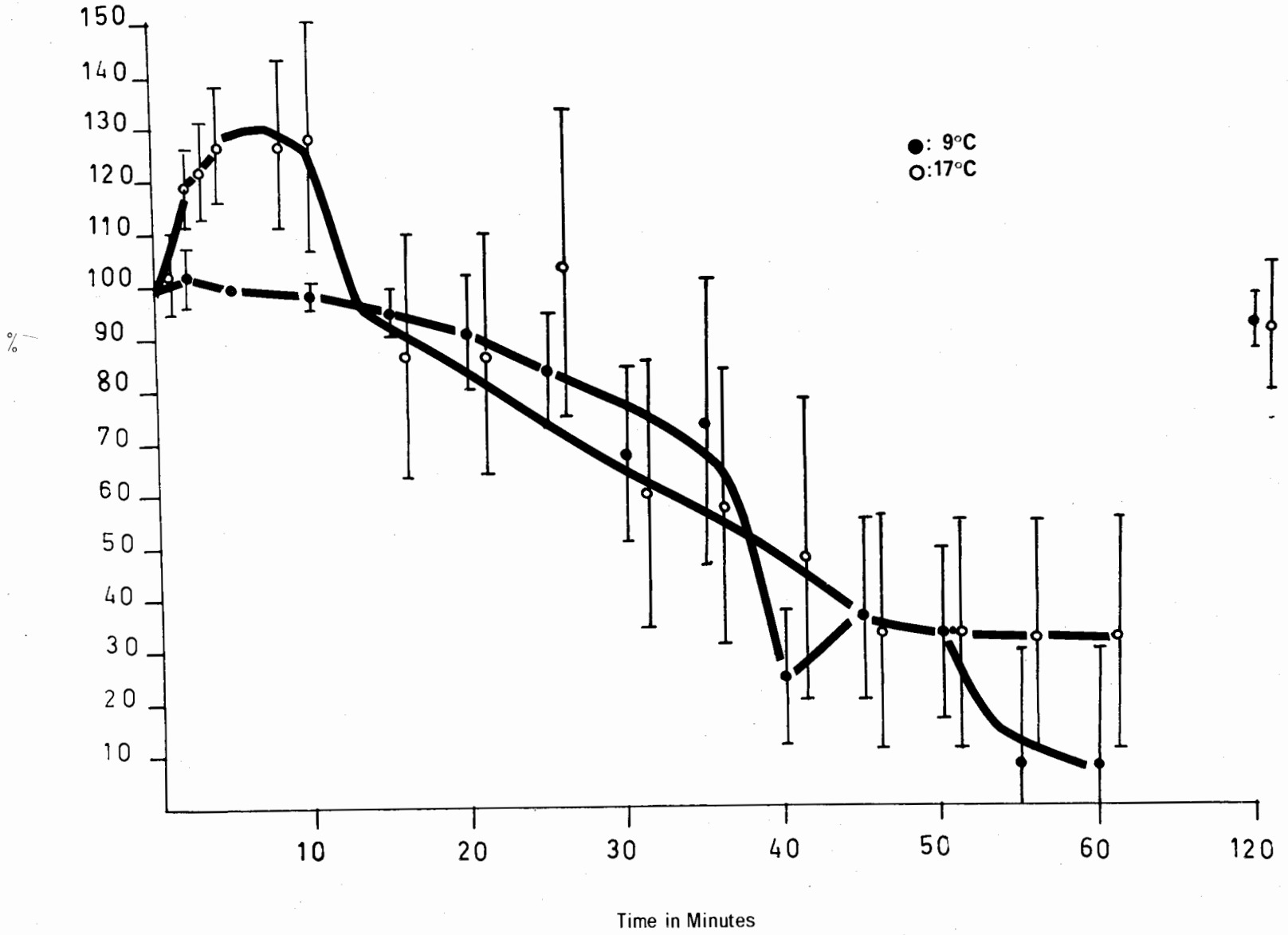

150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
%
●: 9°C
○:17°C
10
20
30
40
50
60
120
Time in Minutes

Figure 13: Effect of 75 mg/l M.S. 222 on the heart rate expressed as percentage of the resting values of rainbow trout at 9° and 17° C. Resting values are considered to be 100%.

Number of fish for each of the two groups: 10.

Values at extreme right were taken 60 minutes after the end of the experiment.

Bars: Represent Standard Errors

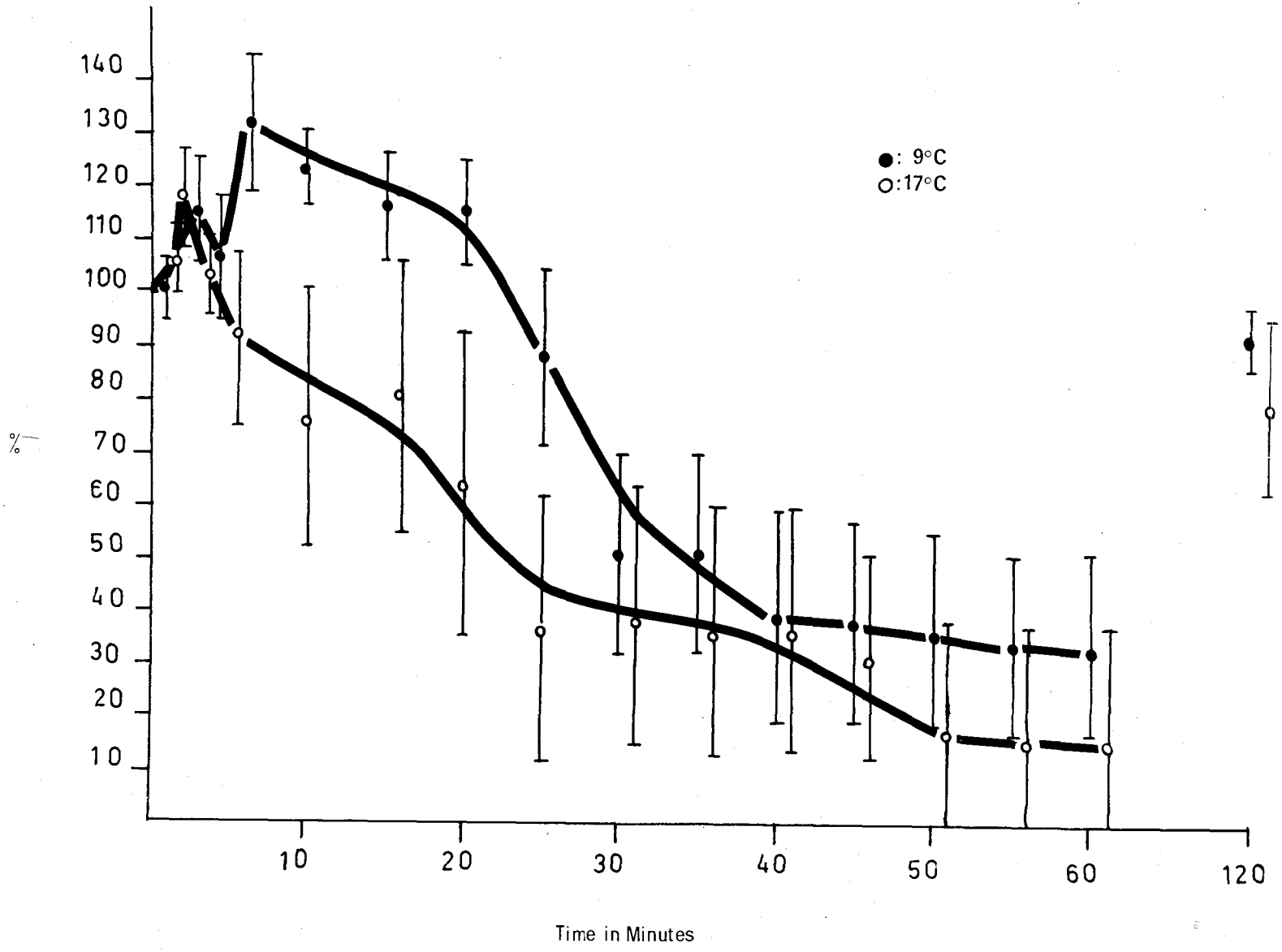
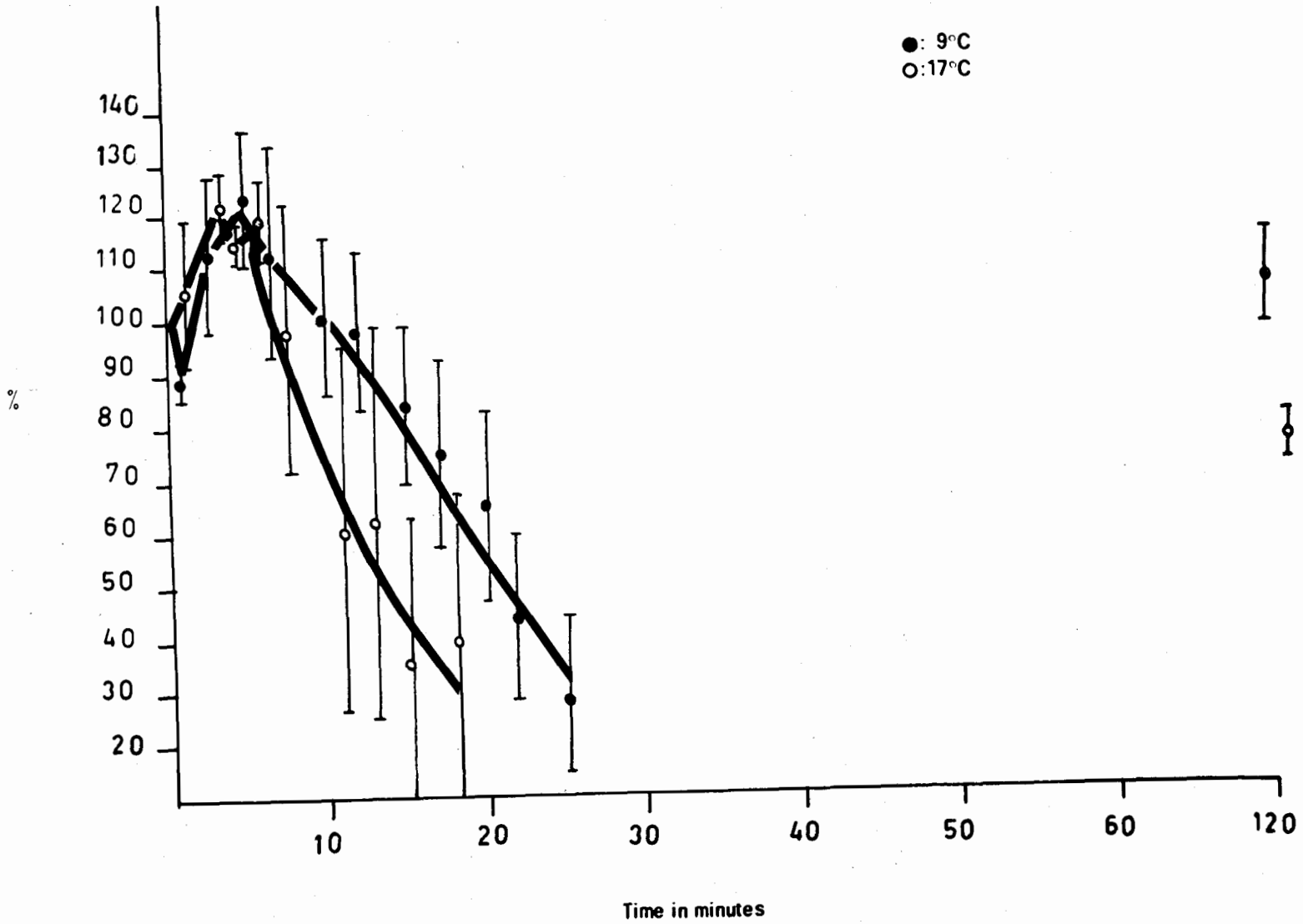

●: 9°C
○:17°C
%
140
130
120
110
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
120
Time in Minutes

Figure 14: Effect of 100 mg/l M.S. 222 on the heart rate expressed as percentage of the resting values of rainbow trout at 9° and 17° C. Resting values are considered to be 100%.

Number of fish for each of the two groups: 10.

Values at the extreme right were taken 60 minutes after the end of the experiment.

Bars: Represent Standard Errors


●: 9°C
○:17°C
%
140
130
120
110
100
90
80
70
60
50
40
30
20
10
20
30
40
50
60
120
Time in minutes

cavity has reached its least volume). As the cavity expands during the "mouth opening" phase, the pressure falls rapidly to zero. The pressure then becomes slightly negative to allow reopening of the oral valves. This only lasts for a very short period of time until the mouth opens sufficiently to bring the pressure back to zero.

a) Effect of handling:

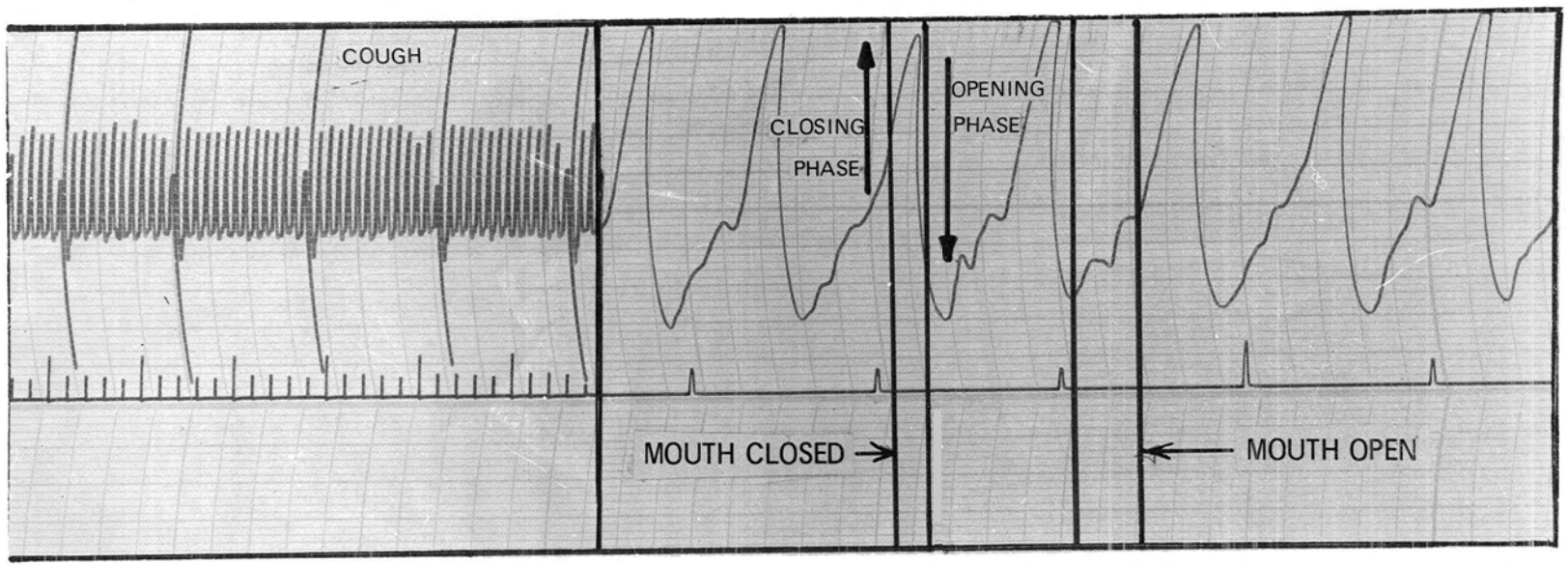
Following handling of the trout, in the manner previously described, we noted a marked increase in the breathing rate (Figure 5). There were no changes in the amplitude of the breathing movements, however, except during the actual handling and a period of about ten minutes thereafter while the fish swam around and made several attempts to jump out of the tank. A return to resting values was observed some 60-90 minutes after handling.

b) Effect of temperature:

Temperature did not affect the breathing rate as markedly as it affected the heart rate. An increase in ambient temperature of the medium from 9° to 27° C caused the rate to increase to 160% of its resting value (Figure 6). There was an initial increase in rate at 13° C to approximately 120% of the resting value. There was no further change until 19° C when the rate increased. Values higher than 160% of the

Figure 15: Typical recording of the respiratory movements of the rainbow trout.

Temperature 9° C.


COUGH
CLOSING
PHASE
OPENING
PHASE
MOUTH CLOSED
MOUTH OPEN

resting respiratory rate were never observed. The most noticeable changes were in the amplitude of the respiratory movements. The amplitude of the movements doubled once the temperature of resistance (20° C) had been reached. The increase in amplitude is limited by the volume of the buccal cavity and the inertia of the movements. This may explain the tremendous demand placed on the heart to pump extra blood over the gills and the relatively small increase in respiratory rate. During this period of stress, repeated attempts were made by the fish to escape from the tank. Recovery time was from 2-3 hours to 24 hours after the water temperature had gradually been lowered back to 9° C.

c) Effect of M.S. 222:

After the introduction of the anaesthetic into the experimental tank, at concentrations of 25 and 50 mg/l there was no initial increase at either temperature (Figures 16 and 17). There was an increase in the respiratory rates at concentrations of 75 and 100 mg/l of 15 to 35% above the resting values at both 9° and 17° C. Within 5 minutes of the start of the experiments, rates at all concentrations declined. This coincides with Stage 1 of anaesthesia. At this time no noticeable change in the amplitude of the respiratory movements is observed.

Figure 16: Effect of various concentrations on the anaesthetic on the respiratory rate expressed as percentage of the resting values of the rainbow trout. Resting values are considered to be 100%.

Temperature 9° C.

Number of fish for each of the four groups: 10.

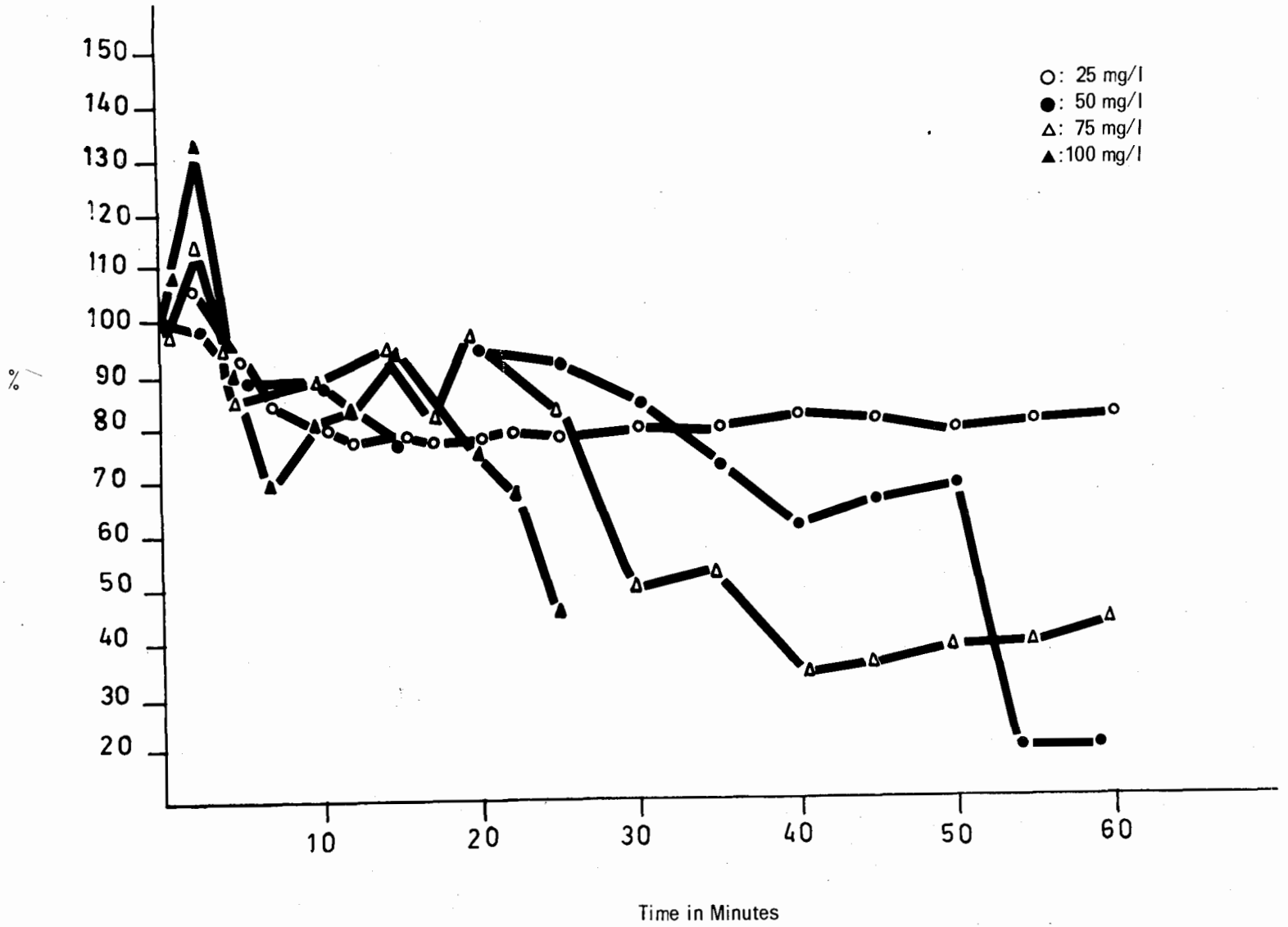
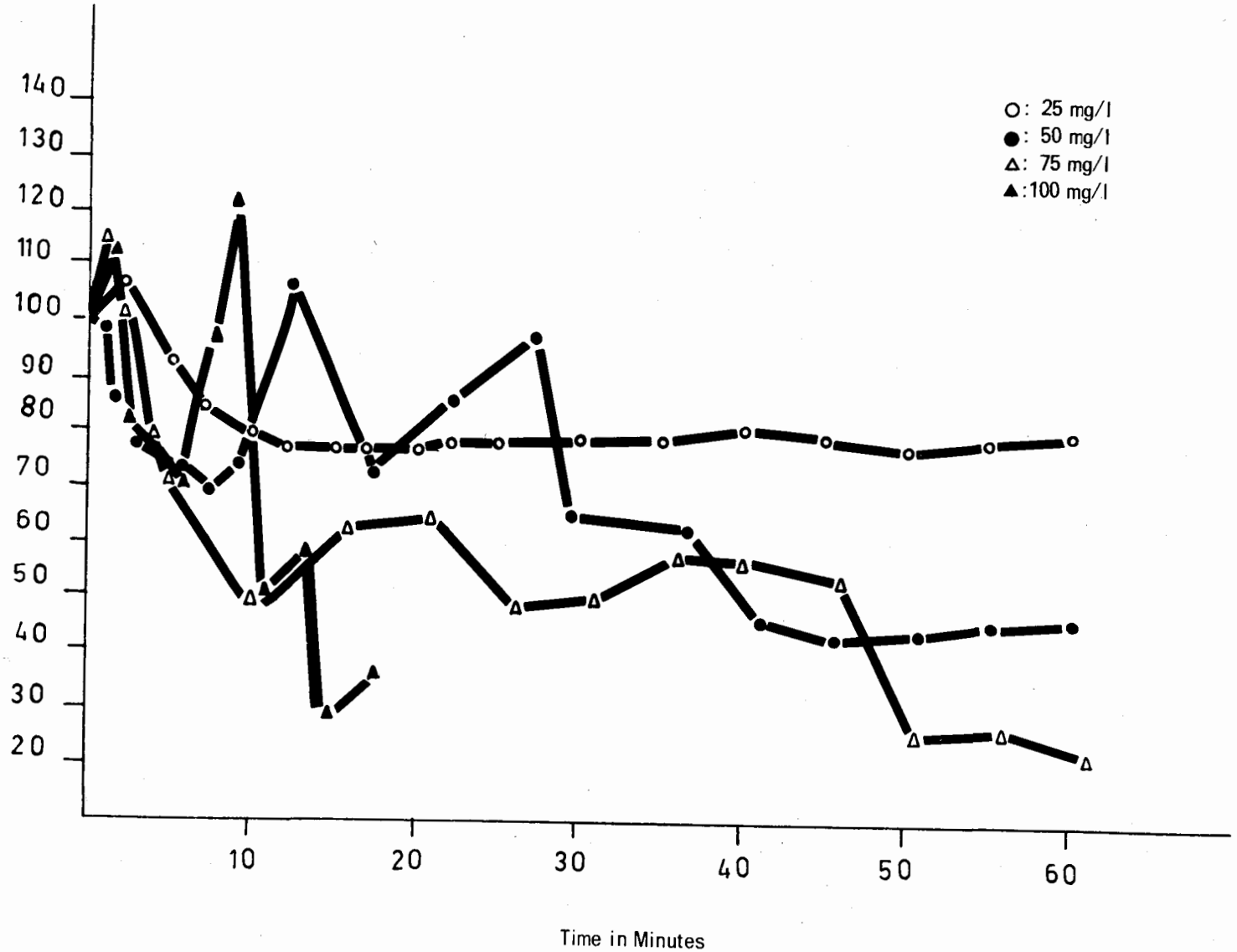

150
140
130
120
110
100
90
80
70
60
50
40
30
20
%
10
20
30
40
50
60
Time in Minutes
○: 25 mg/l
●: 50 mg/l
△: 75 mg/l
▲:100 mg/l

Figure 17: Effect of various concentrations of the anaesthetic on the respiratory rate expressed as percentage of the resting values of the rainbow trout. Resting values are considered to be 100%.

Temperature 17° C.

Number of fish for each of the four groups: 10.


140
130
120
110
100
90
80
70
60
50
40
30
20
%
10
20
30
40
50
60
Time in Minutes
O: 25 mg/l
●: 50 mg/l
△: 75 mg/l
▲:100 mg/l

With 25 mg/l, within ten minutes of the start of the experiments the respiratory rate stabilized at about 75-80% of the resting value, whereas with 50 mg/l and over, the decline was followed by a second increase in rate. This is the transition from Stage 1 of anaesthesia to Stage 2-1. In those fish that underwent respiratory collapse, a rapid decline followed the second peak. This final declining phase was accompanied by a rapid decrease in the amplitude of the respiratory movements to about one fifth of its resting value. At this time there is complete loss of equilibrium, motor and sensory responses (Stage 2-2). Some periods of excitation and brief periods of rapid fluctuation in rate were observed during the transition phase from Stage 2-1 to Stage 2-2. There does not appear to be a significant difference between the effects of the anaesthetic at 9° and 17° C (Figures 18-21; Appendix 1-4). It should be remembered that the resting rates at 17° C were higher by some 15% but that we are considering percentage deviation from the resting values. Respiratory collapse was more frequent and occurred more rapidly at 17° C than at 9° C. No respiratory collapse was observed with 25 mg/l at either temperature (see Table I, Page 31). The recovery from the experimental treatment took longer at 17° C.

Figure 18: Effect of 25 mg/l M.S. 222 on the respiratory rate expressed as percentage of the resting values of rainbow trout at 9° and 17° C. Resting values are considered to be 100%.

Number of fish for each of the two groups: 10.

The values at the extreme right were taken 60 minutes after the end of the experiment.

Bars: Represent Standard Errors

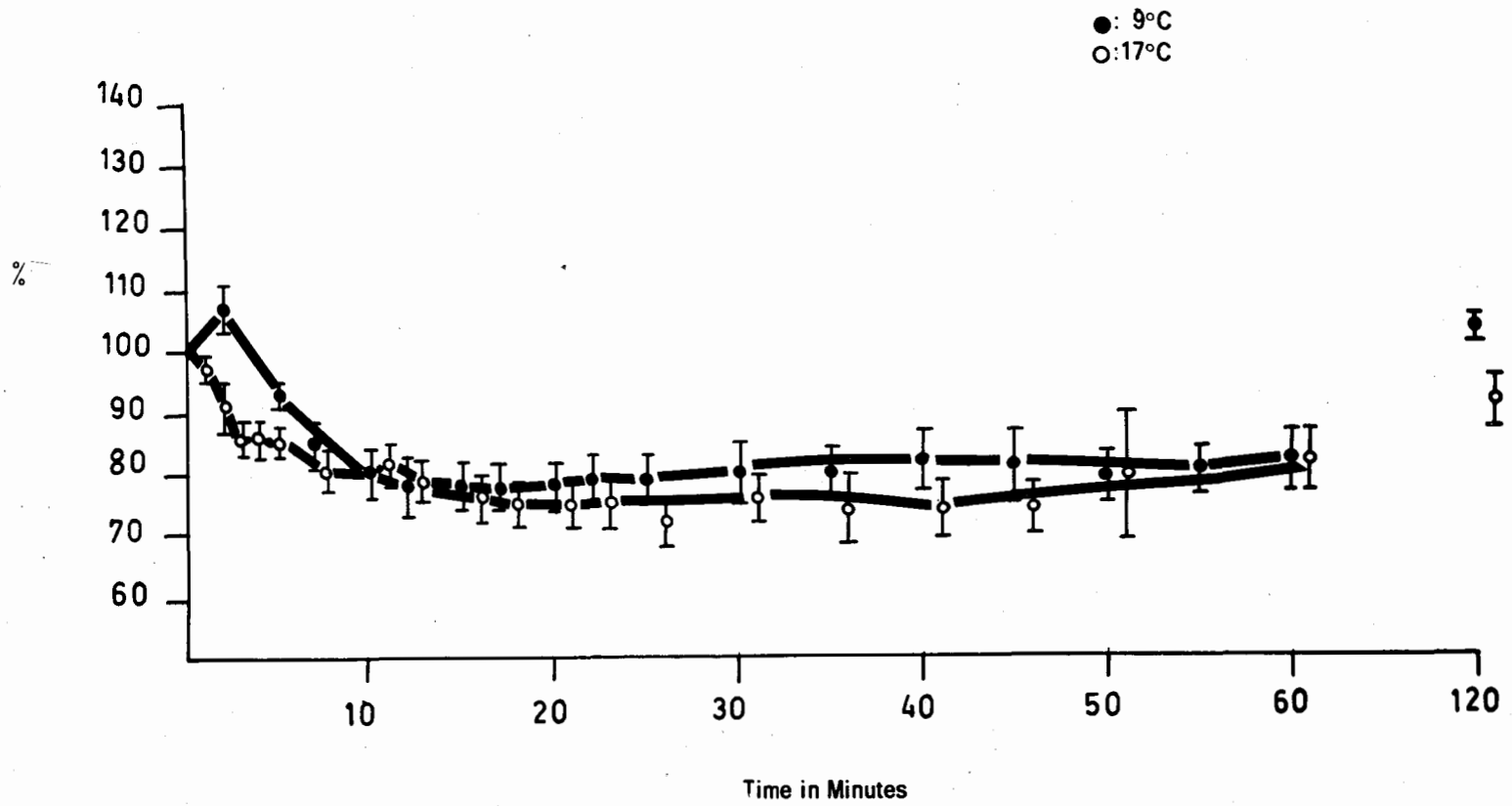

●: 9°C
○:17°C
140
130
120
110
100
90
80
70
60
%
10
20
30
40
50
60
120
Time in Minutes

Figure 19: Effect of 50 mg/l M.S. 222 on the respiratory rate expressed as percentage of the resting values of rainbow trout at 9° and 17° C. Resting values are considered to be 100%.

Number of fish for each of the two groups: 10.

The values at the extreme right were taken 60 minutes after the end of the experiment.

Bars: Represent Standard Errors

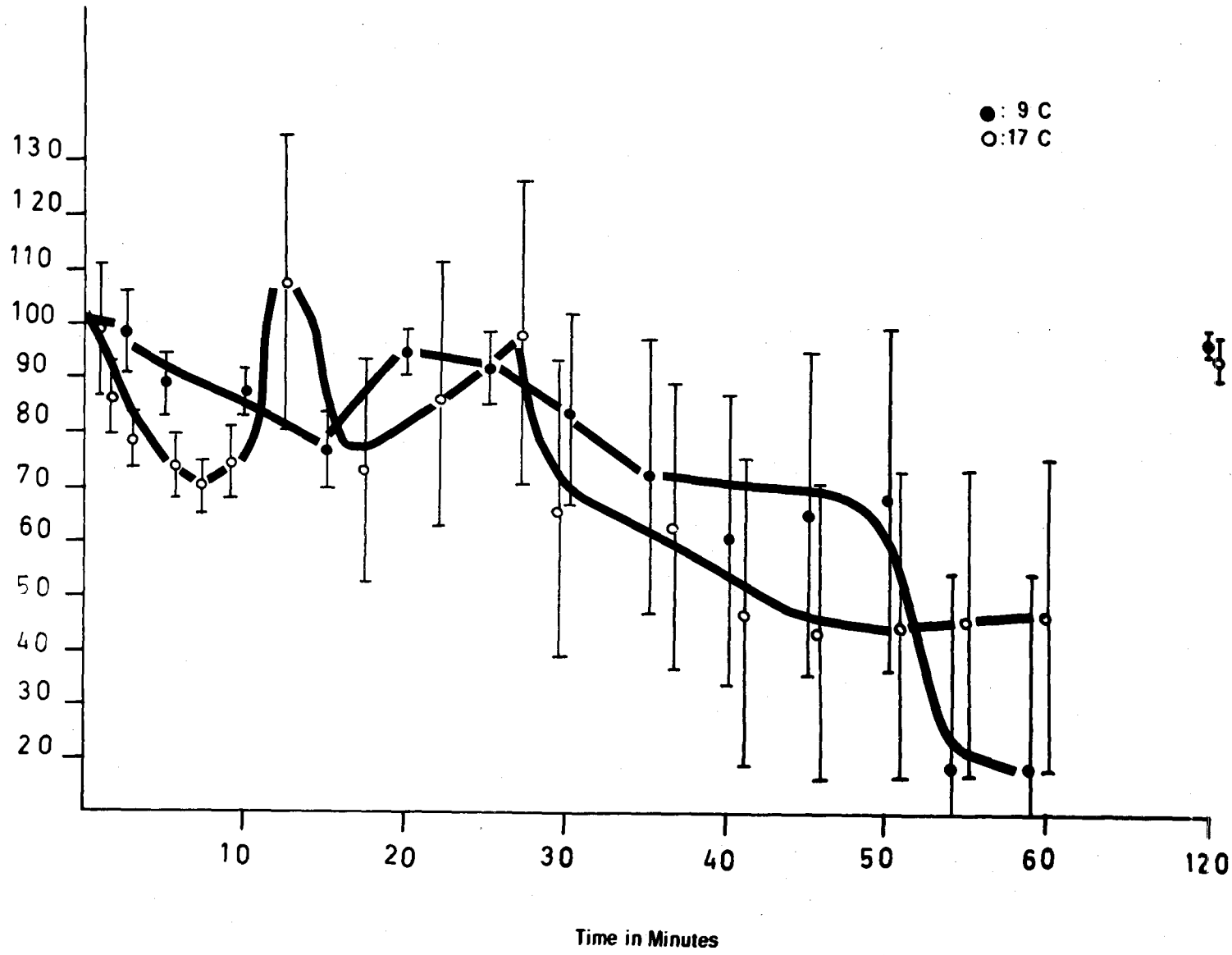
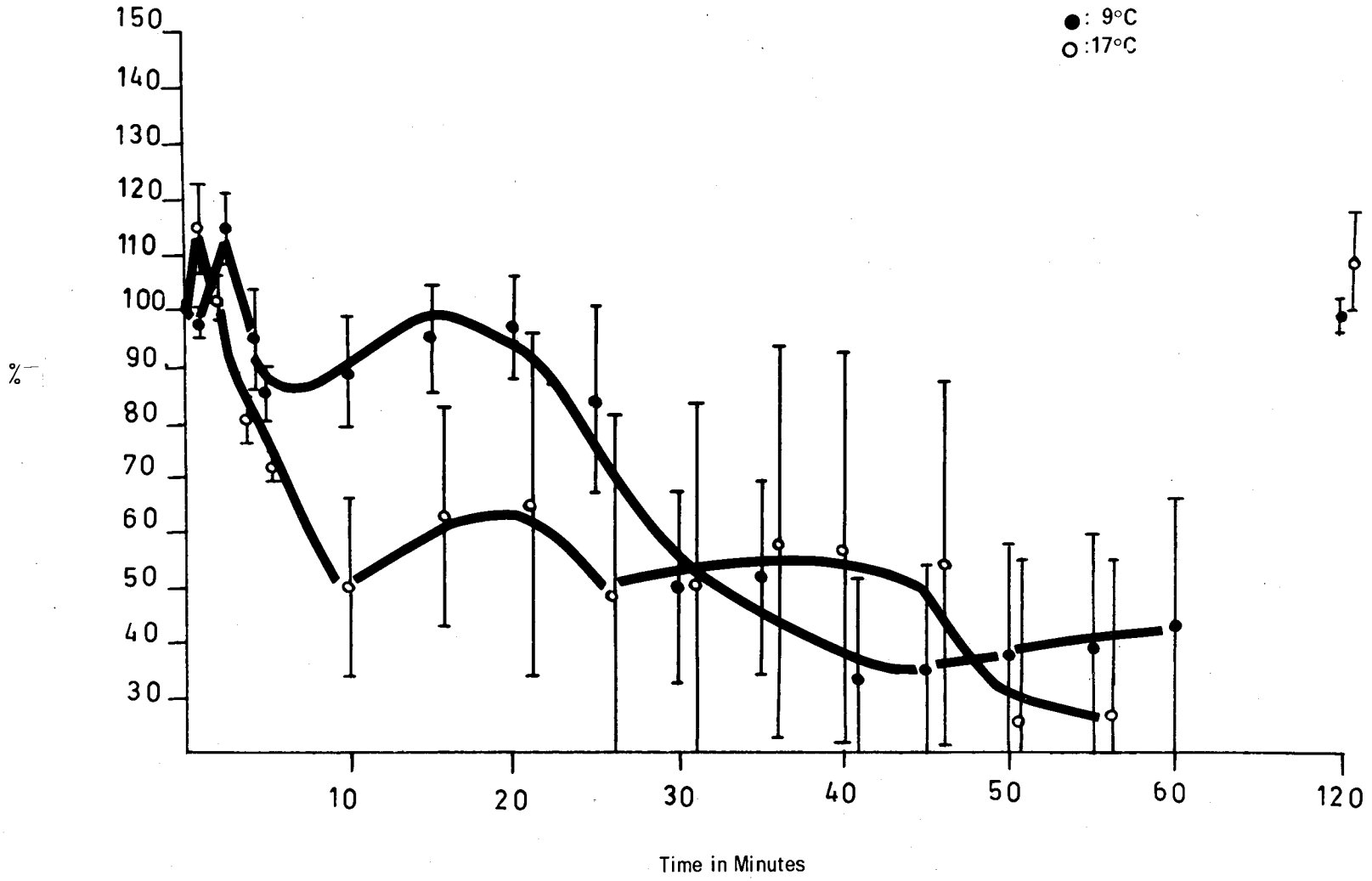

●: 9 C
○:17 C
130
120
110
100
90
80
70
60
50
40
30
20
10
20
30
40
50
60
120
Time in Minutes

Figure 20: Effect of 75 mg/l on the respiratory rate expressed as percentage of the resting values of rainbow trout at 9° and 17° C. Resting values are considered to be 100%.

Number of trout for each of the two groups: 10.

The values at the extreme right were taken 60 minutes after the end of the experiment.

Bars: Represent Standard Errors


●: 9°C
○: 17°C
150
140
130
120
110
100
90
80
70
60
50
40
30
%
10
20
30
40
50
60
120
Time in Minutes

III. Cardiac and Respiration Interrelations.

Correlations between the respiratory movements and cardiac rhythm have been extensively described in the literature (Babak, 1912; Willem, 1921; Lyon, 1926; Lutz, 1930a, b; Satchell, 1960, 1961; Randall and Smith, L. S., 1967). In particular, Satchell (1960) noted that in elasmobranchs the heart-respiration ratio was 1:1, or some multiple of this, with the heart beating in the mouth opening stage of the respiratory cycle. He indicated that this might play a significant role in bringing the maximum amount of blood to the gills at the time of maximum water flow. In the trout, the heart beat has a tendency to occur in the mouth-closing stages when the animal is at rest. A much closer relationship is seen in the anaesthetized fish, when definite correlation can be observed for fairly long periods of time; the heart beat and breathing becoming synchronized in a 1:1 ratio (Figure 22). The length of time during which synchrony was observed increased with the concentration of the anaesthetic at 9° C, from 7% of the total experimental time at 25 mg/l to 70% of the total experimental time at 100 mg/l (see Figure 23). At 17° C the length of time during which synchrony was observed was about 30% of the total experimental time which was very close to the value obtained in the temperature control experiment

Figure 22: Recording demonstrating synchrony between the heart rate and respiration of the anaesthetized rainbow trout.

Temperature 9° C

Chart Speed: 10 mm/sec

Lower Trace: Heart beat and respiratory movements superimposed.

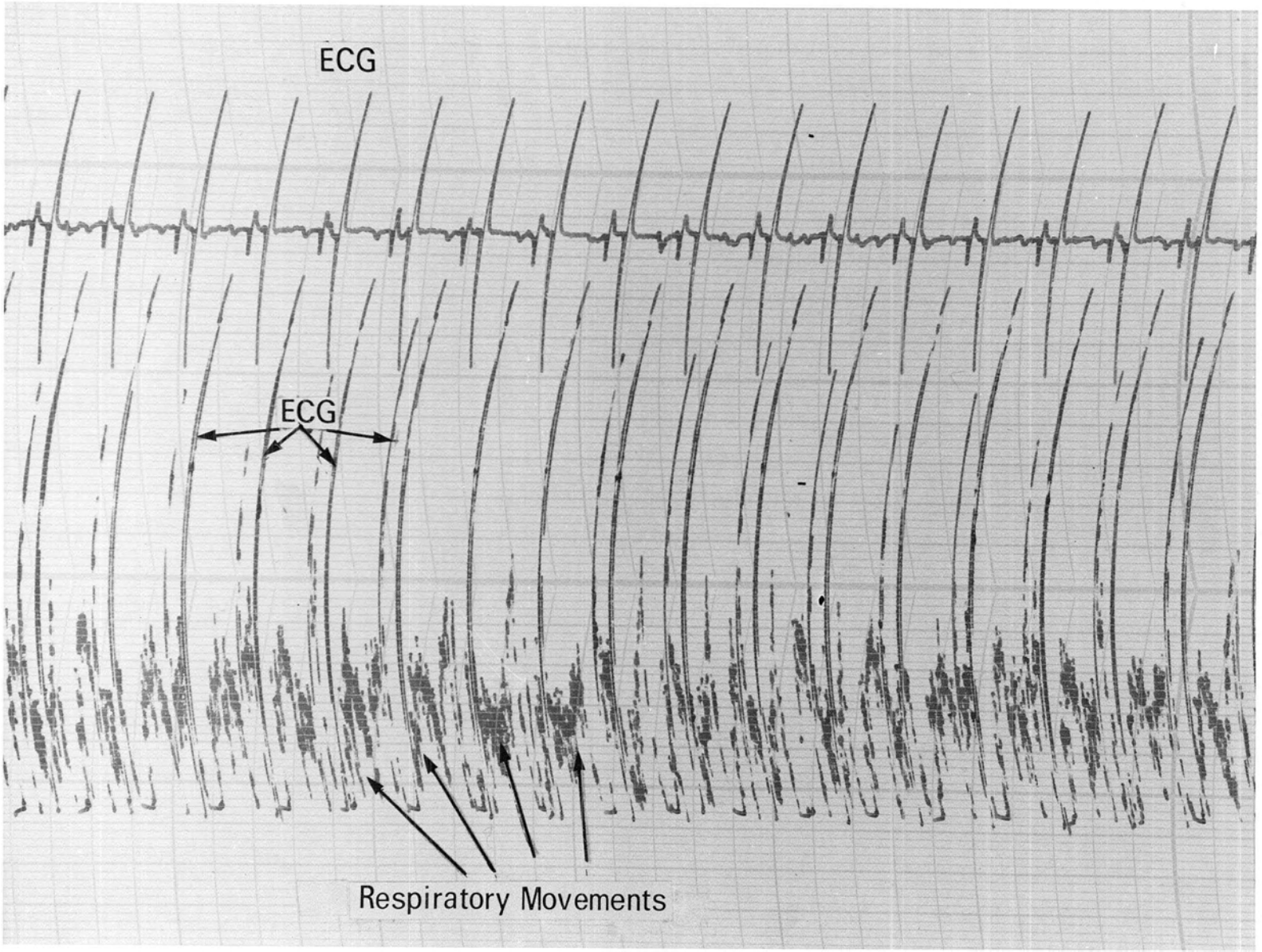

ECG
ECG
Respiratory Movements

Figure 23: Percentage of experimental time synchrony was observed with various concentrations of M.S. 222 at 9° and 17° C.

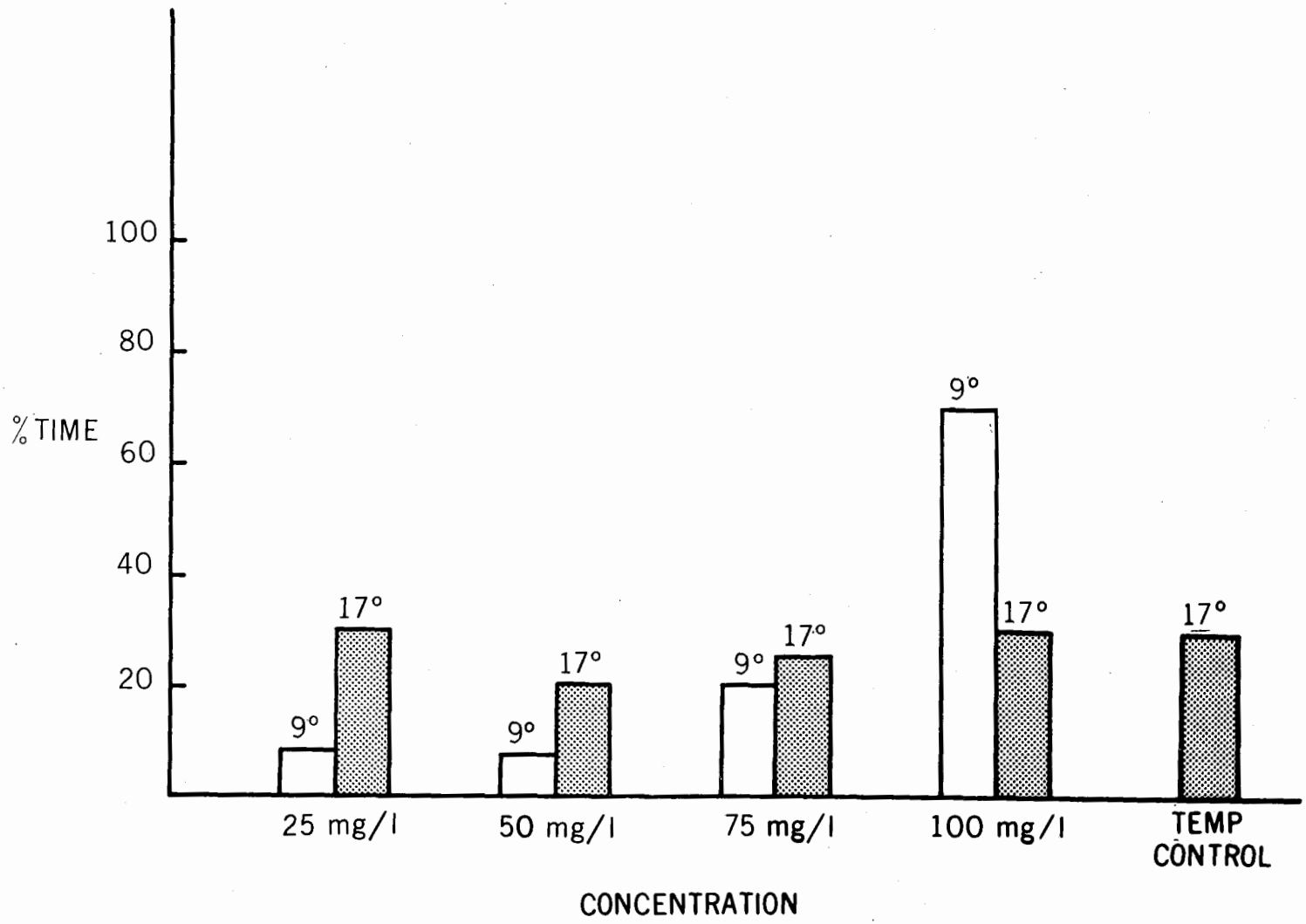

100
80
60
40
20
% TIME
9°
17°
9°
17°
9°
17°
9°
17°
17°
25 mg/l
50 mg/l
75 mg/l
100 mg/l
TEMP CONTROL
CONCENTRATION

TABLE II

Percentage of total experimental time that synchrony was observed in various concentrations of M.S. 222 at 9° and 17° C.

TREATMENT	PERCENTAGE	
	at 9° C	at 17° C
Control	0	30
25 mg/l M.S. 222	8	30
50 mg/l M.S. 222	7	20
75 mg/l M.S. 222	20	25
100 mg/l M.S. 222	70	30

(Table II, Page 49). At this temperature the percentage time did not differ for any of the concentrations. The number of fish in which synchrony was observed increased as the concentration of M.S. 222 increased at 9°C, whereas at 17°C nearly all the fish showed synchrony (Table III, Page 51).

IV. Recordings from the respiratory center.

I was able to record electrical activity related directly to the respiratory movements in those areas described by Shelton (1961) (see Figure 24). I was not able to demonstrate any electrical activity in the areas peripheral to the respiratory center which represented a common pathway from the respiratory center to the heart and myotomes.

TABLE III

Percentage of fish exhibiting synchrony of heart and respiratory rate in various concentrations of M.S. 222 at 9° and 17° C.

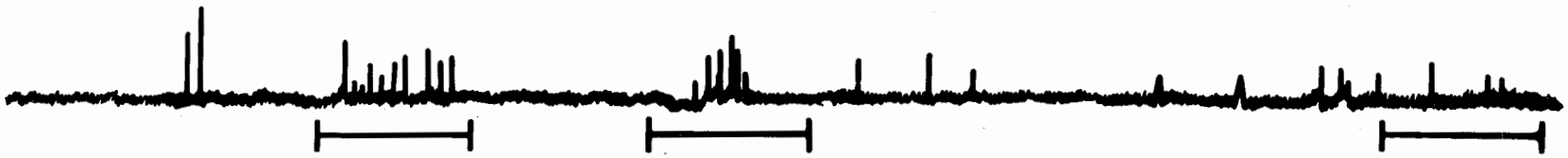
CONCENTRATION OF M.S. 222	PERCENTAGE at 9° C	at 17° C
25 mg/l	20	100
50 mg/l	30	100
75 mg/l	50	90
100 mg/l	60	100

Figure 24: Respiratory activity recorded from the medulla oblongata of the rainbow trout.

|——| indicates bursts of activity related to individual respiratory movements.

Time Base: 5 cm/sec

Voltage: 0.5 mV/cm



DISCUSSION

The methods used in this study are highly sensitive and have several advantages over the earlier mechanical recordings made by Randall (1962) or the water-jacket system of Saunders (1961). To be noted are: (1) the high sensitivity of both the transducers and the recording apparatus; (2) the use of a fluid system with its high sensitivity to pressure changes; (3) the use of cannulae which neither interfere with the flow of water nor with the opercular movements; (4) the freedom given to the animal making it relatively unrestrained and thus, approaching as closely as possible natural conditions.

Our observations on trout during anaesthesia revealed that respiratory and cardiac functions seemed to vary in a consistent manner. The concentrations of anaesthetic required for different levels of narcosis were variable but in general a solution of 25 mg/l M.S. 222 made the animal unreactive to external stimuli after about 30 minutes at 9° C. Concentrations in excess of 25 mg/l caused a rapid loss of equilibrium with no movement except those of breathing. The initial increase in heart rate under anaesthesia was not observed by Randall and Smith (L. S.) (1967). They obtained a steadily decreasing curve from the

onset of the experiment. However, it should be remembered that in the methods they employed fish were restrained, whereas they were free-swimming under my experimental conditions. These differences may be attributed to differences in techniques. The heart beat in my experiments remained fairly regular until respiratory collapse. This again does not concur with the results obtained by Randall and Smith (L. S.) (1967), who, in the tench and trout, observed asystole. It is interesting that in cyprinids (i.e. the tench, goldfish and the carp) these last authors as well as Serfaty et al (1959) noticed that M.S. 222 increased the heart rate. Therefore the difference in effect of M.S. 222 between salmonids (represented by the trout) and cyprinids may suggest differences in their means of regulating cardiac output. Increased cardiac output can be brought about by either increasing the rate or the stroke volume or both. It may be that the cyprinids increase cardiac output by increasing the heart rate whereas salmonids arrive at this same condition by increasing their stroke volume. The respiratory rate and amplitude of the trout also showed marked changes in the presence of M.S. 222. The decline in respiratory rate usually preceded that of the heart rate. It would seem therefore that the heart is less susceptible to the anaesthetic

effects of M.S. 222.

Artificial ventilation of the gills after respiratory collapse nearly restores resting values of the cardiac rate (as well as E. C. G. structure) in the trout. This is a very interesting phenomenon because it has been postulated that there are chemoreceptors on the gills (Hughes and Shelton, 1962). They consider these receptors an important factor in the regulation of gill ventilation. From this one might conclude that the rate of gas exchange rather than the flow of water across the gills is the primary regulatory mechanism. Support for this possible chemical regulation of the respiratory movements comes from a study of DeKock (1963). He has demonstrated the existence of a large number of taste-bud like structures fully exposed to the "respiratory currents" and he considered these to be significant in the control of breathing in fish. The present study, however, notes the possibility that the structures observed by DeKock are pressoreceptors. There is no direct evidence yet to demonstrate the presence of either pressure or chemoreceptors but the regularity and the speed at which the change of heart rate occurred after the water flow is passed forcibly over the gills suggests that at least one of the control mechanisms of gill ventilation may include presso-

receptors.

There did not appear to be any significant difference in the effect of M.S. 222 at 9° and 17° C when these effects are expressed as percentage deviations from the resting values for both the heart and respiratory rates. It does however take the fish longer to recover from the effects of the anaesthetic at 17° C. It is very difficult to place any meaningful explanation to this but in general it appears that as the conditions become less favourable an increase in the amount of time synchrony can be seen. If this were so we would expect longer periods of synchrony at 17°C than at 9°C. Although this holds true for the lower concentrations, it does not for the higher concentrations. Furthermore, the period during which synchrony was observed at 17°C was fairly constant at all concentrations of M.S. 222, and was, moreover, near the value obtained in the temperature control experiments. It appears therefore, that here some other metabolic functions affect the brain centers and mask the effects of the anaesthetic. This may be due to the fact that there is a period of metabolic recovery superimposed on the period of recovery from the anaesthetic. Tiffeneau and Brown (1937) demonstrated that anaesthesia could be induced twice as fast for Gobio species acclimated at 25° C

than for those acclimated at 15° C. These results seem to be in agreement with my results and indicate that chemical reactions are involved in the response of the fish to the anaesthetic as the relevant Q10 approaches the value 2. This would mean that induction of anaesthesia would occur twice as fast at temperatures of 10° C higher. The temperature effect must be related to metabolic changes of the organism rather than to physical characteristics of the anaesthetic because the Q10 related to physical properties is in the order of 1.2.

The dose-response relationship of the anaesthetic is largely independent of the temperature used in the experiment. At both temperatures, the initial increases in the rates reached higher values in the two highest concentrations. Increasing the dosage also decreased the time of induction of narcosis defined as Stage 2 and, increased the resultant depth of anaesthesia as was indicated by the recovery phase.

M.S. 222 may act on the heart via the vagus nerve or some similar pathway because the direct effect of this substance on the perfused heart of the trout is a decrease in frequency of the beat (Randall, 1962). It has been shown that although the passage of water over the gills of teleosts is more or less continuous, a maximum flow is reached during the mouth closing

phase (Randall and Smith, J. C., 1967). Randall (1966) has, moreover, demonstrated that a burst of activity in the vagus prevents the heart from beating at times other than during maximum water flow over the gills. These types of relationship would be in agreement with Satchell's postulate that the significance of the relationship between heart beat and respiratory movement is to bring the maximum amount of blood to the gills at the time of maximum water flow (Satchell, 1961). It appears reasonable that a close relationship should exist in fish between breathing and heart rates. Because of the high density and low oxygen content of the medium, fish such as the trout may use up to 20% of their oxygen consumption for the process of gas exchange itself (Hughes and Shelton, 1962). Indeed, much of the literature deals with studies of the ways by which fish adjust their respiratory processes to changes in the environment in order to lessen this rather high respiratory cost and thereby approach the most efficient system. Any discrepancy between the capacity of the water stream to bring oxygen to the exchanging surfaces and the blood stream to take it away would mean a serious waste of effort on the part of the fish. Although in teleosts synchrony does not appear to be a commonly occurring phenomenon as for instance in elasmobranchs, a close relationship between heart

output and ventilation volume could nevertheless exist. Both functions depend on two independently varying parameters: amplitude and frequency of the movements (rate), and therefore the relationship may be complex and variable from time to time and in different individuals. The fact that the unanaesthetized fish must be at rest and undisturbed for synchrony to occur suggests that in the unanaesthetized animal a similar mechanism as in the anaesthetized animal is acting. This would be in agreement with the synchrony between the breathing movements and the movements of the trunk and of the pectoral fins in the slightly anaesthetized goldfish, described by von Holst (1934a, b). As an explanation of this phenomenon, he suggested that "automatic cells" in the central nervous system were responsible for the generation of a basic rhythmic process which would affect several centers in the brain. This view was opposed by Lissman (1947). He maintained that there was no evidence to show that limited afferent inflow constitutes an essential part of a mechanism which maintains locomotory rhythms, either as a pacemaker or by keeping the central excitatory state at an appropriate level. In my opinion it is reasonable to conceive of a generating center or oscillator similar to the one proposed by von Holst. The center, under natural conditions, may be

inhibited while under conditions of anaesthesia for example, this inhibition may be blocked, thereby making the oscillator active. We know from the work of Shelton (1959, 1961) that the respiratory center in teleosts is diffuse. Therefore we could think in terms of a recruitment of normally inhibited neurons in the peripheral area of the respiratory center following conditions of hypoxia or anaesthesia. Probably musculature other than that normally associated with breathing is affected by the rhythmicity of the center. We would have to consider a spreading of rhythmic waves over various centers probably in the outermost part of the respiratory center when the excitatory level is very low. The existence of synchrony between heart beat and electrical activity in the respiratory center of curarized fish shows that the relationship is not due to a feedback from the respiratory musculature but is generated by impulses within the C. N. S. (Serbenyuk, 1959). Shelton (1959) has shown that the respiratory center in teleosts must be situated caudally to the region where the fifth and seventh cranial nerves emerge from the brain and that these two nerves must be intact for respiration to continue. Thus, although the basic rhythmic process in the C. N. S. may be independent of the sensory nervous system, it is not possible to determine whether

the autonomous activity recorded in an isolated brain can result in normal breathing movements. We do not know to what extent this activity in the isolated brain can be regarded as normal and we can therefore question the validity of relevant E. E. G. recordings. We may record a rhythm of electrical discharges coinciding with the breathing rhythm (as seen in Figure 24), while in fact other neurons may be of primary importance in the generation and regulation of breathing. The main consideration therefore, must be given to the fact that a rhythm does occur, and whether this rhythm reflects a pattern in the respiratory center. There is some evidence from curarized fish that ventilation could be controlled by autonomous pacemakers in central neurons under certain conditions (Serbenyuk, 1959). He observed a secondary rate being initiated in the respiratory center after curarization which was slower than the normal breathing rate and very regular. We could imagine that the activities of these pacemakers are normally modified by reflexes set in action by sensory impulses arising from chemical and pressure stimuli. Lutz (1930) suggested that there must be a close relationship between centers in the medulla oblongata which are concerned with respiratory movements and heart rate. Satchell (1960) considers that any coordination between the

branchial pumps and the heart if effected reflexively. However it should be remembered that he was dealing with a fish in which synchrony occurred naturally and that entirely different nervous pathways may prevail in the elasmobranchs. Several factors may affect fish respiratory activity. The simplest method of control would be one in which the whole network is driven by pacemakers which are intrinsically active. It is very unlikely that any pacemaker is a single cell or a discrete group of cells because localized destruction within the respiratory center was unsuccessful in stopping normal breathing (Randall and Smith, L. C., 1967). We could suppose that the inspiratory neurons are tonically active, omitting a continuous train of impulses unless they are somehow inhibited or stimulated. Stimulation could be generated by the removal of inhibition. Under normal conditions these inhibitions, stimulations or disinhibitions could come from a variety of sources: for example, expiratory neurons, extra-medullary centers such as the pneumotaxic center (although in fish there seems to be little evidence for the dependence of the medulla respiratory center on extra-medullary influence), or the inhibitory inflow of the vagus, or from interactions within central nervous centers themselves. Assuming that the

afferent components in the respiratory nerves have an inhibitory action on the respiratory center, and that these are removed by the introduction of the anaesthetic, we can understand the rise in respiratory rate due to the cessation of the rhythmic sensory inflow from proprioceptors, in other words inhibitory impulses would no longer act upon the respiratory center. Then due to lack of proprioreception the automatic center takes over and we obtain synchrony. When the center is also overcome by the anaesthetic another increase in the respiratory rate occurs, until finally the whole brain will be overcome and respiratory and cardiac collapse occurs. The heart continues to beat for a few minutes after respiratory collapse because of its myogenic nature.

SUMMARY

1. The heart rate of the rainbow trout remains fairly stable under anaesthetic conditions until such time as respiratory collapse occurs. The heart and respiratory rates are affected by the anaesthetic M.S. 222 and the variations can be related to the various stages of anaesthesia. The respiratory rate is the first to be affected indicating that the respiratory center and probably brain centers in general, are more sensitive to the effects of the anaesthetic than the heart.

2. The most suitable concentration of anaesthetic for physiological experimentation on rainbow trout appears to be 25 mg/l although it is evident that ideally the animal should be unrestrained and unanaesthetized in order to obtain experimental results approaching most closely the natural condition.

3. There is no significant difference between the effects of the anaesthetic at 9° and 17° C. Temperature does not appear to be a critical factor in the effectiveness of the anaesthetic although there are some variations in the rates due to increasing temperature.

4. Synchrony between respiratory movements and heart beat can be induced in the trout under conditions of anaesthesia or by raising the temperature of the medium. It appears that synchrony can be induced by any of the factors which tend to make the medium less favourable to the fish. Duration of synchrony increases with dosage of M.S. 222 at 9°C. But at 17° C the time during which synchrony was observed was independent of the concentration, and was the same as in the temperature control experiments. It seems therefore that temperature plays a more important role than the effects of the anaesthetic.

5. Cardiac collapse follows respiratory collapse, but forcibly ventilating the gills during the period of myogenic activity of the heart restores a heart rate very near the resting rate. Therefore it is assumed that some chemical or pressure mechanism resident in the buccal cavity also controls the heart rate.

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APPENDIX 1

Variance between the means. 25 mg/l, 9° and 17° C.

	Respiratory Rate		Heart Rate	
Time (min.)	F ratio	5%	F ratio	5%
2.5	8.29*	4.88	0.49	4.88
5	3.75	4.21	0.00	4.88
7.5	0.76	4.21	0.93	3.87
10	0.10	3.97	6.17*	3.87
12.5	0.01	3.87	1.29	3.87
15	0.10	4.21	0.87	3.87
17.5	0.23	4.21	0.30	3.87
20	0.52	3.87	0.00	4.21
22.5	1.67	4.21	0.32	4.21
25	1.97	4.88	1.22	4.21
30	0.58	4.21	2.78	4.21
35	1.18	4.88	1.74	4.88
40	1.11	4.95	0.78	4.95
45	1.05	4.39	3.52	4.39
50	0.00	4.39	1.08	6.26
60	0.00	4.39	1.38	4.39
120	12.86**	4.88	10.93**	4.88

*: represent values larger than 5%

APPENDIX 2

Variance between the means. 50 mg/l, 9° and 17° C.

	Respiratory Rate		Heart Rate	
Time (min.)	F ratio	5%	F ratio	5%
2.5	1.37	5.05	5.11*	5.05
5	3.29	4.95	7.41*	5.05
10	0.07	4.53	3.09	4.53
15	0.76	6.16	2.61	6.16
20	0.15	4.95	0.03	4.39
25	0.00	4.05	5.27*	4.53
30	0.29	4.39	0.06	4.95
35	0.06	4.95	0.87	4.95
40	0.13	4.95	0.59	4.95
45	0.30	4.95	0.01	4.95
50	0.33	4.95	0.00	4.95
55	1.25	4.39	1.12	4.39
60	0.65	4.39	1.27	4.39
120	0.54	4.39	0.01	4.95

*: represent values larger than 5%

**: represent values larger than 1%

APPENDIX 3

Variance between the means. 75 mg/l, 9° and 17° C.

	Respiratory Rate		Heart Rate	
Time (min.)	F ratio	5%	F ratio	5%
1	4.97*	3.58	1.12	4.15
2	1.48	4.06	0.02	3.22
4	1.63	4.15	0.09	4.15
5	2.72	4.74	3.47	4.06
10	4.90*	4.06	5.00**	4.06
15	2.60	4.06	2.32	4.06
20	1.49	4.06	4.09*	4.06
25	1.14	4.06	2.04	4.06
30	0.01	4.06	0.15	4.06
35	0.02	3.22	0.15	4.06
40	0.42	3.22	0.01	4.06
45	0.24	3.22	0.07	4.06
50	0.17	4.06	0.50	4.06
55	0.28	4.06	0.11	4.06
60	0.52	4.06	0.56	4.06
120	1.45	3.22	0.81	4.06

*: represent values larger than 5%

**: represent values larger than 1%

APPENDIX 4

Variance between the means. 100 mg/l, 9° and 17° C.

	Respiratory Rate		Heart Rate	
Time (min.)	F ratio	5%	F ratio	5%
1	0.17	4.76	1.89	4.53
2	14.80**	3.86	0.04	3.63
5	1.30	6.00	0.00	3.63
7.5	6.90*	3.86	0.20	8.81
10	1.32	6.00	1.78	6.00
12.5	0.67	6.00	1.19	6.00
15	5.13	6.00	2.28	6.00
17.5	1.74	6.00	0.84	6.00
90	1.85	3.63	8.26*	6.00

*: represent values larger than 5%

**: represent values larger than 1%

APPENDIX 5

Calculations of Standard Error: Heart Rates.

Concentrations of M.S. 222:	25 mg/l		50 mg/l		75 mg/l		100 mg/l	
Temperature:	9° C.	17° C.	9° C.	17° C.	9° C.	17° C.	9° C.	17° C.
Time								
2	114± 4	109± 7	102± 6	119± 7	116±10	118±10	113±15	110± 9
5	108± 6	109± 7	100± 0	118± 7	132±13	92±17	124±13	122± 9
7	102± 2	112± 8	100± 0	126±16	128± 8	85±20	113±19	97±25
10	100± 3	109± 7	99± 1	128±21	124± 7	77±25	101±15	60±34
12.5	101± 3	109± 7	97± 3	123±17	120± 4	76±25	98±15	62±36
15	105± 3	111± 6	95± 4	86±22	117±10	81±27	84±15	35±39
17.5	104± 4	107± 5	94± 5	86±22	117±10	67±31	75±18	40±39
20	109± 7	109± 5	91±11	86±22	116±10	64±29	66±18	-
22.5	115±9	108± 6	87±11	86±22	95±12	60±27	45±16	-
25	118±10	104± 5	84±11	104±29	88±17	38±24	29±15	-
30	119±10	98± 6	68±17	60±26	51±19	39±25	-	-
35	119±12	100± 9	74±27	58±26	51±19	36±23	-	-
40	119±12	105±10	25±13	48±29	40±20	36±23	-	-
45	113± 4	100± 6	37±17	34±22	38±19	31±19	-	-
50	111± 5	100± 9	34±16	33±22	37±18	17±24	-	-
55	114± 7	100± 9	9±23	33±22	35±17	15±24	-	-
60	110± 8	99± 5	8±23	33±22	34±17	15±24	-	-
120	95± 4	78± 3	92± 5	91±12	92± 6	79±16	106± 9	76± 4

APPENDIX 6

Calculations of Standard Error: Respiratory Rates.

Concentrations of M.S. 222:	25 mg/l		50 mg/l		75 mg/l		100 mg/l	
Temperature:	9° C.	17° C.	9° C.	17° C.	9° C.	17° C.	9° C.	17° C.
Time								
2	107±4	91± 4	99± 8	87± 7	115± 6	103± 4	133± 6	82±14
5	93±2	85± 2	89± 6	74± 6	85± 5	72± 3	90± 7	73±13
7	85±4	80± 3	89± 6	70± 5	87± 7	64±14	69± 9	121±21
10	80±4	81± 3	88± 3	92±17	89±10	50±16	80±11	51±29
12.5	78±5	79± 3	80± 5	108±27	92± 9	53±17	83±13	59±34
15	78±4	76± 3	77± 7	69±11	95±10	63±20	93±14	29±37
17.5	78±4	75± 3	95± 4	73±20	95±10	54±25	81±16	36±34
20	78±4	75± 4	95± 4	86±24	97± 9	65±31	75±19	-
22.5	79±4	75± 3	94± 4	86±24	89±12	76±36	67±22	-
25	79±4	72± 3	92± 7	94±27	84±17	49±32	45±23	-
30	80±5	76± 3	84±18	66±27	50±17	51±32	-	-
35	80±4	74± 5	73±25	64±26	53±18	58±36	-	-
40	82±5	74± 5	61±27	47±28	34±18	57±36	-	-
45	81±6	74± 4	67±30	44±27	36±19	54±33	-	-
50	79±4	79±10	69±31	45±26	38±20	26±35	-	-
55	81±3	80±10	19±31	47±28	43±23	22±37	-	-
60	82±4	82±5	19±31	46±28	43±23	22±37	-	-
120	103±2	91±3	98±2	95± 4	99± 3	109± 9	102± 2	117±17

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