ANAESTHESIA IN ABALONE, *Haliotis* midae

THESIS

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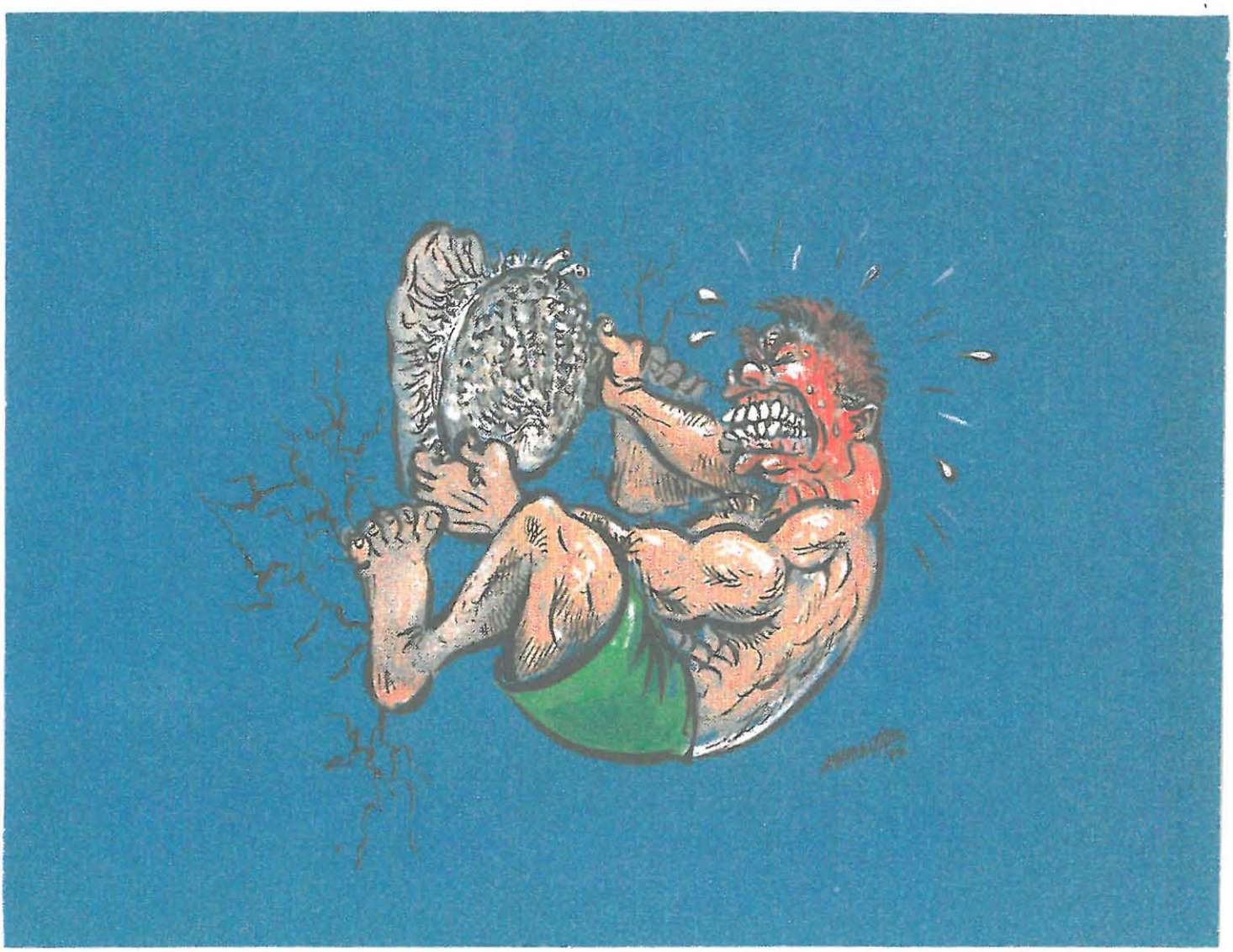

THE PROBLEM

TABLE OF CONTENTS

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ABSTRACT

The principle aim of this study was to isolate a chemical for the "safe anaesthesia" of abalone under commercial farming conditions. "Safe anaesthesia" implied that the anaesthetic had no immediate detrimental or long term sublethal effect on the abalone, that it was safe for the farmer, the consumer and the environment.

Four chemicals, magnesium sulphate ($MgSO_4$), ethylenediamine tetra-acetic acid (EDTA), 2-phenoxyethanol and procaine hydrochloride were shown to effectively inhibit the *in vitro* contraction of isolated tarsal muscle of *Haliotis midae*. This identified them as potential anaesthetics for abalone.

Since abalone, like any other aquaculture species, would be subject to frequent size-sorting during the grow-out period, size related dosage tables were developed for the four chemicals at a temperature of 18°C. Dosage tables were also developed for benzocaine and carbon dioxide (CO_2). Three size classes (5-15, 20-50 and 60-90 mm shell length (SL)) of abalone were considered. Only three of the six chemicals, viz. $MgSO_4$, 2-phenoxyethanol and CO_2, met the criteria of an effective abalone anaesthetic in that they effected rapid and mortality-free anaesthesia. The other three chemicals caused mortalities and were considered to be unsuitable for commercial scale anaesthesia.

Temperature related dosage tables were then developed for $MgSO_4$ and CO_2. $MgSO_4$ concentrations and CO_2 flow rates for effective anaesthesia in abalone were found to be inversely related to temperature.

The three size classes of *H. midae* were intermittently exposed to $MgSO_4$ and 2-phenoxyethanol anaesthesia for an eight month period to determine the effect of the anaesthetics on growth rate. Because of an increased resistance to the efficacy of 2-phenoxyethanol and high monthly mortalities it was concluded that this chemical was unsafe and unsuitable for commercial use. $MgSO_4$, on the other hand, had no effect on growth of abalone and no significant effect on the rate of mortality.

$MgSO_4$ also had no measurable effect on *H. midae* muscle ultrastructure and, by implication had no effect on flesh texture. The use of $MgSO_4$ as an anaesthetic would, therefore, not affect marketability. Moreover, no magnesium residues were found in *H. midae* muscle tissue after short term or intermittent long term exposure to $MgSO_4$ anaesthesia.

It was found that the three size classes of *H. midae* used in this study could be safely exposed to the recommended $MgSO_4$ concentrations for up to 40 minutes without any mortalities. This is more than adequate for routine farming procedures. Medium size abalone (20-50 mm SL) were also safely exposed to 14 g.100 ml^{-1} $MgSO_4$ for up to 6 hours without any mortalities.

The results have shown that $MgSO_4$ was undoubtedly the best chemical that was evaluated for anaesthesia of *H. midae* in this study . It fulfils the requirements set forth by the U.S.A. Food and Drug Administration (FDA) in that it is safe for the abalone, the farmer, the consumer and the environment.

CHAPTER 1
INTRODUCTION

Abalone are gastropod molluscs belonging to the order Archeogastropoda, the family Haliotidae and the genus *Haliotis* Linnaeus, 1758 (Fallu 1991). Approximately one hundred species of *Haliotis* occur worldwide, and are found on the coasts of almost every continent and on many of the islands in the Pacific, Atlantic and Indian Oceans (Cox 1962, Hahn 1989a, Shepherd *et al.* 1992). Of the six species of abalone which occur in South Africa only *H. midae* occurs in sufficient quantities to warrant commercial exploitation (Newman 1967, Barkai & Griffiths 1986, Hahn 1989a, Tarr 1992, Hecht 1994).

Abalone occur intertidally and to depths in excess of 400 m (Lindberg 1992). Their preferred habitat is crevices on rocky reefs and overhangs, which provide protection from light and predators (Crofts 1929, 1937, Fallu 1991). The South African abalone, *H. midae* is found in the shallow sublittoral zone between St. Helena Bay on the west coast, and Port St. Johns on the east coast (Field *et al.* 1977, Hecht 1994). Juvenile *H. midae* ($<$ 50 mm shell length (SL)) are found in the intertidal zone under boulders and small rocks. Larger animals ($>$50 mm SL) generally occur on shallow in-shore reefs (Newman 1966, 1967). There is some discrepancy as to the exact depths at which the majority of larger *H. midae* occur (Newman 1969, Barkai and Griffiths 1986, Lindberg 1992, Tarr 1992). However, it can be safely said that *H. midae* occurs abundantly from the low tide mark down to depths of about 20 m. According to Newman (1969) *H. midae* is more concentrated in areas where the average annual sea temperature lies between 15 and 17°C, i.e. between Cape Hangklip and Quoin Point, although Hecht (1994) argues that it is not temperature which determines the distribution of abalone, but the availability of suitable seaweeds.

After the Californian abalone, *H. rufescens*, the South African species is one of the largest haliotids (Newman 1968, Tarr 1992). It would appear that abalone in the Western Cape grow at a slower rate, attain a greater size and a higher maximum age, and attain sexual maturity at a larger size than those in the Eastern Cape (Newman 1968, Wood 1993). These differences can most probably be ascribed to lower sea temperatures along the Western Cape coast.

The natural diet of abalone changes with each stage in the life-cycle. The larvae are

considered to be lecithotrophic (Fallu 1991, Manahan & Jaeckle 1992), but more recent research has shown that they require an exogenous source of organic carbon to supply additional energy not supplied by the yolk (Manahan & Jaeckle 1992). After metamorphosis, from the pelagic to the benthic or spat stage, the abalone feed on benthic diatoms which occur on coralline algae (McShane & Smith 1988, McShane 1992), whereafter they change their diet to macro algae (Fallu 1991, Shepherd & Steinberg 1992). Abalone feed on both drift and attached seaweeds and feed mainly at night (Wood 1993). The natural diet consists of some 18 species of algae of which the most important are *Ecklonia maxima*, *Laminaria pallida*, *Plocamium corallorhiza* and *Ulva* spp. (Newman 1969, Field *et al.* 1977, Barkai & Griffiths 1986, 1987, Tarr 1992, Wood 1993, Britz *et al.* 1994). Green (*Ulva* spp.) and brown (*Ralfsia expansa*) algae are taken in larger proportion by the smaller sub-boulder cryptic animals (Barkai & Griffiths 1986, Wood 1993), while red seaweeds (in particular *P. corallorhiza* and *Hypnea spicifera*) are preferred by exposed large animals in the Eastern Cape (Wood 1993) and kelp, *E. maxima*, in the Western Cape (Newman 1969, Field *et al.* 1977, Barkai & Griffiths 1986, 1987).

Abalone have been exploited for hundreds of years for food and for making ornaments and the manufacture of jewellery (Barkai & Griffiths 1986, Shepherd *et al.* 1992). It has only been in the last 35 years that abalone fisheries have evolved globally and become economically important in many countries (Shepherd *et al.* 1992). Only 22 *Haliotis* species are commercially important (Hahn 1989a, Fallu 1991). The major fisheries are found in Mexico (ca. 34% of world production), Japan (29%), Australia (20%), South Africa (6%), the United States (5%), Korea(3%) and New Zealand (3%) (Hooker & Morse 1985). Before 1984, Mexico, Japan and Australia were each landing over 5000 metric tons annually (Chew 1984).

The South African commercial abalone fishery started in 1949 (Newman 1967, Tarr 1992), and a substantial commercial fishery has been in existence between Cape Agulhas and Cape Point since the 1950s (Newman 1966, Barkai & Griffiths 1987). A small abalone fishery now also exists at Hamburg along the South East Cape coast (Wood 1993). The development of the South African abalone fishery has been reviewed by Newman (1967) and Tarr (1992). No catch records are available for the first few years of the fishery (Newman 1967). Records of the estimated landings based on production quotas are only available from 1953 onwards (Tarr 1992, R. Tarr, SFRI, pers.comm.). Whole mass quota figures are available from 1983,

when TAC's were implemented (R. Tarr, SFRI, pers. comm.). Recreational catch data for *H. midae* are only available for 1992, 1993 and 1994 and although no figures are available, poaching of *H. midae* has definitely increased in the last two years (R. Tarr, SFRI, pers. comm.). The available catch records since the beginning of the fishery are summarized in Figure 1.1.

Intensification of commercial fishing activities, the development of more practical diving gear and increased poaching has resulted in the collapse of wild fisheries in many parts of the world, especially over the past two decades (Fallu 1991, Tarr 1992). This had led to the farming of abalone in most of those countries which supported abalone fisheries, as a means of enhancing over-exploited wild stock and to satisfy market demand (Hahn 1989b, Shepherd *et al.* 1992).

Abalone aquaculture was pioneered in Japan in the 1950s and 1960s (Hahn 1989c). At present, Japan, the U.S.A. and Taiwan are the most advanced as far as abalone culture technology is concerned (Hecht & Britz 1990). Approximate figures of wild-harvested and cultured abalone produced during 1991 in these countries are given in Table 1.1. Australia, New Zealand and South Africa are now also on the threshold of producing farmed abalone.

The development of abalone culture technology in South Africa only began in earnest in 1989/1990 (Cook 1991). Three research institutions, the University of Cape Town, the Earth Marine and Atmospheric Technologies Division (EMATEK) of the Council of Scientific and Industrial Research (CSIR) and Rhodes University, initiated research programmes in collaboration with the private sector to develop the technology for the culture of *H. midae* (Hecht & Britz 1992, Britz *et al.* 1994). The spawning and settling technology for *H. midae* was largely perfected by the University of Cape Town and EMATEK (Hecht 1992), while at Rhodes University research was undertaken on the development of a nutritionally complete, formulated feed, the development of a weaning diet as well as identifying the optimal environmental requirements for the intensive farming of abalone (Dixon 1992, Hecht 1992, Britz *et al.* 1994, Knauer 1994). The research and development phase for abalone farming in South Africa has now reached the point at which most of the pilot plants are being upgraded for commercial scale production.

Abalone farming practices such as size-sorting, maintenance of proper densities, transfer

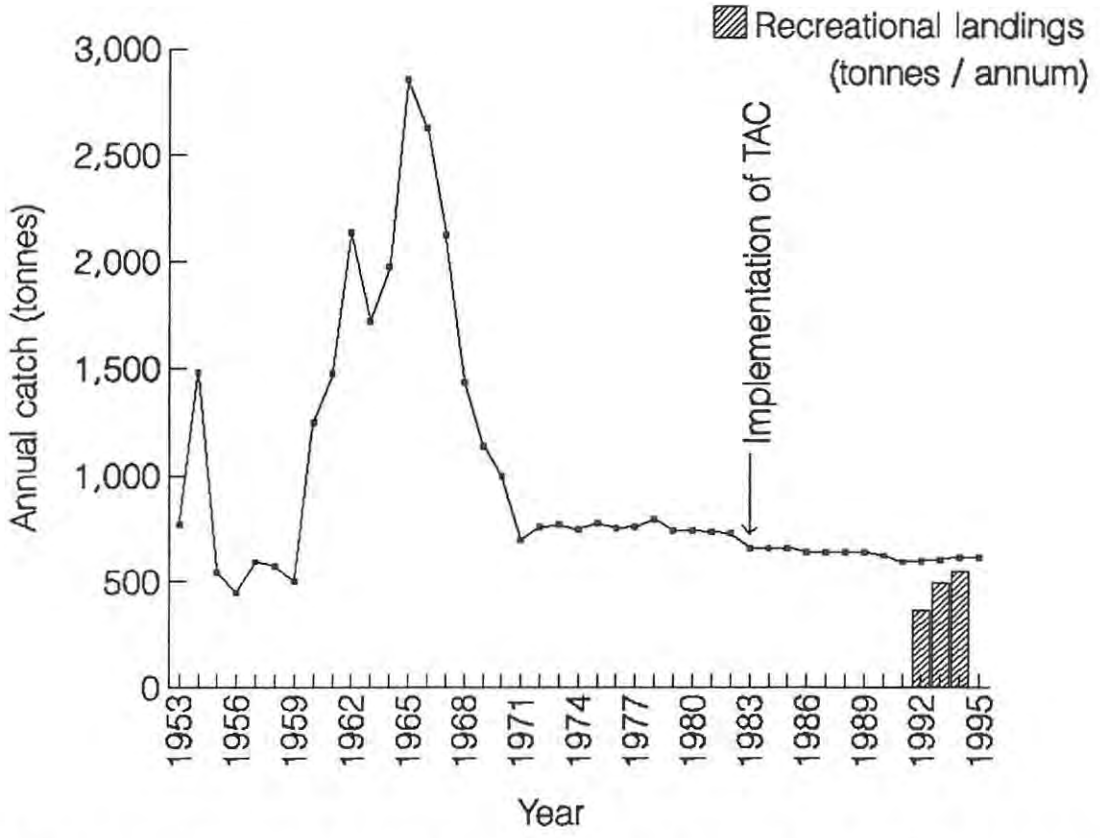


Figure 1.1. Estimated commercial landings of *Haliotis midae* (whole mass) before and after implementation of TAC's based on production quotas from 1953 to 1995 (R. Tarr, SFRI, pers. comm.). Recreational figures for *H. midae* (whole mass) from 1992 to 1994 are also presented.

between tanks, system maintenance and harvesting require the periodic removal of abalone from their holding tanks (Hahn 1989d, Tegner & Butler 1989, Shepherd *et al.* 1992, Tong *et al.* 1992). Abalone possess a large muscular foot which functions as an adhesive organ (Fretter & Graham 1962), which makes it possible to clamp the shell down tightly onto the substratum. This makes it extremely difficult to remove the animal from the substratum (Barnes 1987). Considering the number of abalone in a commercial sized farm, mechanical removal by hand would be logistically impractical. Moreover, mechanical dislodgement often results in injury and death during routine collection or system maintenance (Genade *et al.* 1988, Hahn 1989d). This is principally a consequence of the slow healing rate of abalone, the absence of a blood coagulation system and an increased probability of bacterial infection and stress (Cox 1962, Armstrong *et al.* 1971, Genade *et al.* 1988). Removal of the abalone from the tanks by anaesthesia or by relaxing their muscles provides an alternative and safer method than mechanical removal.

Table 1.1. Approximate figures of cultured and wild-harvested abalone produced in Japan, Taiwan, USA, Australia and New Zealand in 1991 (Britz 1991).

Country	Wild-harvested abalone (tonnes/annum)	Cultured abalone (tonnes/annum)
Japan	5000	360
Taiwan	not available	250
USA	373	60
Australia	5000	commercial production in experimental stage
New Zealand	1147	commercial production in experimental stage
Mexico	± 600	commercial production in experimental stage
South Africa	615	commercial production in experimental stage

Muscular relaxation and anaesthesia can be achieved by various mechanisms (Lefkowitz *et al.* 1991, Hondeghem & Miller 1992, Neal 1992, Watanabe & Katzung 1992). Firstly, by means of neuromuscular blocking drugs which compete with acetylcholine (ACh) (a neurotransmitter involved in muscle contraction) to bind to its post-ganglionic receptors. These competitive drugs reduce the end-plate depolarizations produced by ACh to a magnitude below the threshold for muscular action potential generation and so cause flaccid paralysis. Secondly, by way of local anaesthetics which block the conduction of impulses along nerves. Thirdly, by way of calcium chelating agents or excess magnesium which inhibits the release of ACh into the synaptic cleft by blocking the influx of extracellular calcium into the nerve terminal. This influx of calcium through voltage-dependent channels into the nerve terminal is essential for the release of ACh into the synaptic cleft at the neuro-muscular junction.

Anaesthetics are also very important in fish culture to produce a wide range of desired effects that vary from mild sedation to complete loss of equilibrium and insensitivity (Marking & Meyer 1985). Anaesthetics are commonly used on fish to aid in spawning, transporting, tagging, marking, surgery and stocking (Sagara & Ninomiya 1970, Marking & Meyer 1985). A wide range of anaesthetics are used in fish. These include tricaine methanesulfonate (MS-222), quinaldine, carbon dioxide (CO_2), carbonic acid, sodium bicarbonate ($NaHCO_3$), 2-phenoxyethanol, methyl pentynol, benzocaine, etodimate, metomidate, chlorotone, chlorobutanol, halothane, methoxyflurane, sodium thiamylal, phenthiazamine, propanidid, salt (sodium chloride), electricity, nicotine and tobacco juice (Booke *et al.* 1978, Ferreira *et al.* 1979, Post 1979, Marking & Meyer 1985, Gilderhus & Marking 1987, Gilderhus 1989, Iwama *et al.* 1989, Mattson & Riple 1989, Gilderhus 1990, Parma de Croux 1990, Gilderhus *et al.* 1991). Of these, only MS-222, CO_2 gas and $NaHCO_3$ are currently registered by the U.S.A. Food and Drug Administration (FDA) for the use as anaesthetics in fish cultured for human consumption (Schnick *et al.* 1979, Gilderhus *et al.* 1991, Stefan 1992). MS-222 is used at a concentration of 15 to 40 ppm active ingredient during live transportation of fish, depending on the species (Collins 1990). The fish cannot, however, be sold for human consumption for 21 days after exposure to the anaesthetic (Collins 1990, Gilderhus *et al.* 1991). Quinaldine, used at a concentration of 15 to 30 ppm active ingredient during live transportation, may only be used on non-food fish. $NaHCO_3$ can be used at concentrations of 142 to 642 ppm for five minutes as a means of introducing CO_2 into the water to anaesthetize fish (Stefan 1992). CO_2 gas can be used for anaesthetic purposes in cold and warm water fish. In Canada, no anaesthetics are approved for use with food fish (Prince *et al.* 1995).

Anaesthetics are used in invertebrate culture for immobilization, surgery on bivalves, pearl insertion in pearl oysters, opening of oyster and scallop shells and sampling of abalone populations (Fujioka 1964 cited in Sagara & Ninomiya 1970, Prince & Ford 1985, Hahn 1989d, Heasman *et al.* 1995). Several substances have been used to induce anaesthesia in invertebrates. Magnesium sulphate ($MgSO_4$), magnesium chloride ($MgCl_2$), urethane, veterinary Nembutal (sodium pentobarbitone), menthol, chloral hydrate, benzocaine, eucaine hydrochloride have been used in bivalve molluscs such as the European flat oyster, *Ostrea edulis* (Culloty & Mulcahy 1992) and scallop, *Pecten fumatus* (Heasman *et al.* 1995). $MgSO_4$, $MgCl_2$, MS-222, chloral hydrate, ethanol, urethan, ether, halothane, enflurane and isoflurane have been used in freshwater and marine gastropod molluscs (Kaplan 1969,

Girdlestone *et al.* 1989). $MgCl_2$, isobutyl alcohol, methyl pentynol and chloretone have been used for cephalopods (Messenger *et al.* 1985) and lobster, *Homarus americanus* (Foley *et al.* 1966).

Several anaesthetics have been evaluated for abalone aquaculture (Hahn 1989d). Potassium chloride (Hamada 1965 cited in Sagara & Ninomiya 1970) and chlorpromazine (Enomoto 1969 cited in Sagara & Ninomiya 1970) were shown to be unsuccessful anaesthetics. Sagara and Ninomiya (1970) investigated the anaesthetic effect of ethyl carbamic acid, $MgSO_4$, chloral hydrate and sodium diethylbarbituric acid on juvenile *H. gigantea*. They found ethyl carbamic acid (0.5 and 1% solutions) to be the best anaesthetic for *H. gigantea*. However, carbamic acid is a known carcinogen, which makes it unsafe. $MgSO_4$ (20 and 30% solutions) was found to be safe for *H. gigantea*, but the time required for complete anaesthesia was considered by them to be too long (12 minutes). Chloral hydrate had a rapid onset of action, but recovery times were too long. Moreover, the animals could only be exposed to chloral hydrate for 5 minutes whereafter they had to be returned to clean seawater to prevent high rates of mortality. Sodium diethylbarbituric acid (2% solution) showed potential, but recovery times were too long (viz. 2.5 hours). CO_2 has also been tested for juvenile abalone (Sugiyama & Tanaka 1982). Diethyl carbonate and benzocaine (ethyl p-aminobenzoic acid) have also been found to be successful anaesthetics for small abalone (Prince & Ford 1985, Hahn 1989d). Benzocaine is currently used for abalone anaesthesia in Japan, New Zealand and Australia (Hahn 1989d, Tong *et al.* 1992, Anonymous 1994). However, there are several problems surrounding the use of benzocaine for abalone anaesthesia. High mortalities have been observed by some abalone farmers in South Africa and benzocaine is insoluble in water and has to be dissolved in alcohol prior to its use.

The efficacy of anaesthetics differ from species to species and an anaesthetic which results in mortalities in one species might be very effective and not lethal in another. Therefore, even though $MgSO_4$ was not considered to be ideal for scallops, *H. gigantea* or lobster it was one of the substances considered for *H. midae* in this study.

Restrictions on the use of anaesthetics in aquaculture relate not only to the ineffectiveness of the substances, but also to the legality of their use (Marking & Meyer 1985). In the development of an anaesthetic, the discovery of an effective compound is relatively easy compared with meeting the requirements of the U.S.A. FDA for its registration. Since most

anaesthetics are absorbed through the gills, residues are likely to accumulate in the tissue unless allowance is made for adequate depuration time. The FDA requires that any compound used to anaesthetize fish destined for human consumption must either be excreted or metabolized before fish are consumed. Abalone farmers must be aware of the registration status of chemicals and avoid the use of unregistered ones, as this may seriously jeopardise the international marketing of their product.

The aim of this study was to find a suitable, alternative muscular relaxant or anaesthetic to benzocaine for mass anaesthesia of *H. midae*. The following conditions were set; (i) the substance should have no short term detrimental effect on abalone, (ii) should have no long term sub-lethal effects on growth and (iii) there should be no residues in abalone muscle tissue which could be harmful to the consumer. Residues of the substance in animals should preferably be inactive, if at all present, by the time it reaches the consumer (Brown 1989, Larocque *et al.* 1991). Furthermore, the substance should be easily obtainable and relatively inexpensive.

Four anaesthetics and muscular relaxants were selected and their *in vitro* effect on isolated *H. midae* tarsal muscle was investigated (Chapter 2). These were procaine hydrochloride, $MgSO_4$ (interferes with the release of ACh from the nerve terminal), EDTA (ethylenediamine tetra-acetic acid which is a calcium chelating agent) and 2-phenoxyethanol (mechanism of action not well documented). Procaine, a local anaesthetic, is safe for medicinal use by humans. It is available over-the-counter as Salusa 45 tablets (50 mg procaine hydrochloride per tablet) and therefore easily obtainable. It is used for the treatment of fatigue in elderly people due to its antidepressant properties in the central nervous system. $MgSO_4$ commonly known as epsom salts, is an unscheduled laxative. It is also used as a fertiliser as well as in the food industry and is easily obtainable. EDTA is a chelating agent which precipitates calcium (Reynolds 1982). This is a pure chemical reaction and should have no significant adverse effects on the consumer. 2-Phenoxyethanol has been used widely in fish (Ross & Ross 1984, Marking & Meyer 1985, Gilderhus & Marking 1987, Yamamitsu & Itazawa 1988, Iwama *et al.* 1989, Mattson & Riple 1989, Teo *et al.* 1989, Teo & Chen 1993). Although it is not classified as a hazardous substance its MSDS (Material Safety Data Sheet) from Sigma Chemicals Corporation (U.S.A./Canada) states that it is harmful if swallowed and can cause ocular and respiratory irritation (M.A. Carelaru, Sigma Chemicals Corporation, St. Louis, U.S.A., pers. comm., T. Hall, Whitehead Scientific Laboratories,

Cape Town, pers. comm.). Safety precautions also require the user to wear protective clothing. The aim of this investigation was to identify the most affective anaesthetic agents.

Since abalone, like any other aquatic species, would be subjected to frequent size sorting the next stage of the project was to develop a size-related dosage table for $MgSO_4$, 2-phenoxyethanol, EDTA and procaine hydrochloride (Chapter 3). Two additional anaesthetics were also evaluated. These were benzocaine and CO_2. The efficacy of anaesthetics can vary widely with temperature (Gilderhus & Marking 1987). This necessitated the development of a temperature-related dosage table for $MgSO_4$ and CO_2, since these two chemicals proved to be most effective (Chapter 4). Abalone have very slow growth rates. To evaluate the long term effects of intermittent anaesthesia on growth rate, an eight month growth trial was undertaken with 2-phenoxyethanol and $MgSO_4$ (Chapter 5). This was followed by an investigation into the effect of $MgSO_4$ on the ultrastructure of the muscle tissue (Chapter 6). The principle reason for undertaking this was to establish whether anaesthesia affects flesh texture which in turn, would affect marketability. Since residues of anaesthetic substances used in aquaculture should preferably be inactive, if at all present, by the time the product reaches the consumer, it was necessary to test for $MgSO_4$ residues in the muscle tissue (Chapter 7). Finally, given the nature of on-farm activities, it was deemed necessary to determine the length of time that abalone could be exposed to $MgSO_4$ before short or long term effects became manifest (Chapter 8). The study is summarized in Chapter 9.

CHAPTER 2

THE *IN VITRO* EFFECTS OF FOUR ANAESTHETICS ON ISOLATED TARSAL MUSCLE OF *HALIOTIS MIDAE*

INTRODUCTION

The pedal musculature of abalone is composed of two functionally and structurally distinct regions: the columellar muscle (or the right shell muscle) and the tarsal muscle (or the foot), surrounded by the epipodium. The foot is connected to the shell by means of the columellar muscle (Fretter & Graham 1962, Trueman & Brown 1985, Frescura 1990). The columellar muscle is responsible for effecting major body movements and changes in shape and posture: protraction, retraction, twisting, elevation and lowering of the shell and clamping down onto the substratum. The tarsal muscle is involved in the fine movements of locomotion and food manipulation. The locomotor waves are restricted to the outer edges of the foot and do not pass through the centre portion of the sole where the columellar muscle inserts into it. The tarsal muscle is also responsible for adherence onto the substratum (Trueman & Brown 1985, Frescura 1990). Both of these muscles also play an essential role in righting the body after falling over by moving the foot and epipodium in a flexible manner, attaching itself in part to the substratum and pulling the shell over the top.

Histologically these two regions consist of muscle bundle fibres ensheathed in collagenous connective tissue (Voltzow 1990, Frescura 1990). The columellar muscle rises up as a stout muscular pillar from the centre of the muscular plaque of the foot (Crofts 1929). The columellar muscle consists mainly of thick dorso-ventral muscle bundles which form the long axis of this muscle. In addition, it contains muscle bundles that are orientated perpendicular to the long axis in at least two directions, i.e. radial and circular. The radial muscles are prevalent in the centre, and the circular muscles around the periphery of the columellar region. The tarsal muscle consists of bundles of fibres that are arranged in a three-dimensional network of interconnecting contractile fibres. Sagittal and transverse sections through the foot of *H. kamtschatkana* showed that the thick muscle bundles of the tarsal region are adjacent to the columellar region (Voltzow 1990). From this it is evident that the tarsic region surrounds the columellar region anteriorly, posteriorly and laterally and the columellar muscle inserts on the sole of the epithelium in the central core of the foot. At the ventral and lateral extremities of the foot the tarsal and columellar muscle bundles branch into smaller bundles. These branching systems do not appear to include the ventral surface

of the sole. Instead, this portion of the foot contains isolated or obliquely transverse muscle fibres.

The tarsal muscle contains finer branched muscle bundles and a larger proportion of collagenous connective tissue than the columellar muscle (Voltzow 1990, Frescura 1990). The columellar muscle consists of bundles of muscle fibres wrapped in thin connective tissue sheaths (Voltzow 1990). Virtually no other extracellular connective tissue is present. In the tarsic region, the connective-tissue sheaths become thicker. The bundles of muscle fibres in the tarsic region branch and change direction as they extend from their origin to their insertions. The bundles become finer and finer as they approach the periphery of the foot and become more deeply embedded in the connective tissue of the ventral and lateral extremities. Connective tissue is an essential element of pedal function. The increased volume fraction of connective tissue at the periphery of the foot probably provides the increased flexibility of this region, because the action of each muscle fibre can be amplified by the passive action of the connective tissue around it. The columellar and tarsic regions receive blood from separate branches of the anterior aorta. The dorsal surface of the foot is the most extensively vascularized area.

The tarsal muscle is not as solid as the columellar muscle. The columellar muscle functions as a muscular hydrostat with tightly packed muscle bundles of different orientation antagonising each other (Trueman & Brown 1985, Frescura 1990). The tarsal muscle, however, is a hydrostatic system in which the role of body fluid is intermediate between that of a classic hydrostatic cavity and muscular-hydrostat (Voltzow 1990). The tarsal muscle also has an increased prevalence of sarcolemmal cisternae which enables it to relax more rapidly than the columellar muscle.

Apart from their functional and structural differences, the tarsos also differs physiologically from the columellar region in that it has larger mitochondrial numbers which suggests that it is possibly more oxidative than the columellar region. Clamping down of the shell is a more spasmodic activity whereas adherence onto and movement over the substratum are gradual, smooth actions. However, without the transmission of impulses and therefore action potentials along nerve and muscle cells, muscular contraction cannot take place (Bowman & Rand 1980, Lefkowitz *et al.* 1991, Katzung 1992). Transmission of nerve impulses between synapses, the junctions between neighbouring nerve cells as well as between nerve cells and

muscle cells are modulated by neurotransmitters (Bowman & Rand 1980, Nicaise & Amsellem 1983).

The following neurotransmitters have been identified in molluscs: Acetylcholine (ACh), serotonin (5-hydroxytryptamine), dopamine, glutamate, the neuropeptide FMRFamide and some peptides (Fretter & Graham 1962, Bullock 1965, Jones 1983, Muneoka & Twarog 1983, Nicaise & Amsellem 1983, Walker 1986, Russell & Evans 1989, Frescura 1990). The location and effect of some neurotransmitters have been outlined in Table 2.1. Some of these play an essential role in muscle activity. ACh is known to stimulate muscle contraction in molluscs (Welsh & Smith 1949, Sugi & Yamaguchi 1976, Frescura 1990). It is also known to activate ACh receptors in mammalian smooth muscle (Lefkowitz *et al.* 1991, Katzung 1992).

Carbachol, a synthetic ester of choline, on the other hand, is a direct-acting cholinoceptor stimulant. This means that it mimics the action of ACh on its receptors, and therefore also causes muscular contraction (Bowman & Rand 1980, Taylor 1991, Watanabe & Katzung 1992). It also possesses a weak inhibitory action on ACh uptake into the nerve terminal and thereby increases the availability of ACh in the neuromuscular junction (Bowman & Rand 1980). Carbachol has been shown to produce muscle contraction in *Mytilus edulis* (Muneoka *et al.* 1979).

It was hypothesized that anaesthesia or muscle relaxation would interfere with the functioning of ACh in abalone muscle. Therefore, the aim of this investigation was, firstly, to test the effect of ACh and Carbachol on whole abalone columellar and/or tarsal muscle contraction for further use in isolated organ experiments. Secondly, the contractile response of different sections of abalone tarsal and columellar muscle to carbachol was determined in order to study differences in the contractility of these muscles. Thirdly, the effect of four chemicals (magnesium sulphate ($MgSO_4$), 2-phenoxyethanol, procaine hydrochloride and ethylenediamine tetra-acetic acid (EDTA)), was tested on isolated abalone tarsal muscle to evaluate their potential as suitable anaesthetics or muscular relaxants for abalone. The advantages of these chemicals as potential abalone anaesthetics have been outlined in Chapter 1.

Table 2.1. The effect of some neurotransmitters on molluscan cells.

Neurotransmitter	Location	Effect
ACh	Heart[1,2,3,4]	Inhibition
	Buccal retractor muscle[2]	Relaxation
	Columellar muscle[5]	Contraction
Serotonin	Heart[1,3,6,7,8,9,10,11,12]	Cardioregulation Excitation Positive inotropic and chronotropic effect Increase in aortic systolic, diastolic and pulse pressure
	Adductor muscle[13]	Relaxation
	Gill[14]	Relaxation
	Cilia[15]	Excitation
Dopamine	Heart[11]	Excitation and inhibition
	Gill[14]	Contraction
FMRFamide	Circulation[16]	Regulation of blood pressure
	Heart[16,17]	Inhibition

[1] Welsh & Smith (1949) [2] Bullock (1965) [3] Hill & Welsh (1966)
[4] Jones (1983) [5] Frescura (1990) [6] Muneoka & Twarog (1983)
[7] Russell & Evans (1989) [8] Hill (1958) [9] Hill & Thibault (1968)
[10] Hill (1974) [11] Liebeswar *et al.* (1975) [12] Krajniak & Bourne (1989)
[13] Salanki *et al.* (1980) [14] Ruben & Lukowiak (1984) [15] Walker (1986)
[16] Krajniak & Bourne (1987) [17] Wells (1983)

MATERIALS AND METHODS

Abalone and a limpet species, *Patella oculus*, were collected along the East Cape Coast of South Africa and held in a recirculating system at Rhodes University. The animals were acclimated to laboratory conditions for a period of one month.

The first step of this investigation was to find a suitable neurotransmitter which would stimulate abalone muscle contraction. A preliminary study was therefore performed to test the contractile response of whole tarsal and columellar muscle to ACh. To achieve this, 0.182 g ACh chloride was dissolved in 10 ml of seawater to make up a 1 x 10^{-2} M ACh solution, which was either used undiluted or if lower concentrations were required, the necessary dilutions were made by dissolving the required amount of undiluted ACh solution

in seawater. Fresh ACh solutions were made when required. Three solutions were made up from the stock solution, viz. 1.0×10^{-5} M, 1×10^{-4} M and 1.0×10^{-3} M. This range spans the concentrations which Frescura (1990) used to stimulate columellar muscle contraction in three limpet species, *P. oculus*, *P. vulgata* and *P. barbara*. The musculature of intact abalone was bathed in the three ACh solutions (1.0×10^{-5} M, 1×10^{-4} M, 1.0×10^{-3} M and undiluted (1×10^{-2} M) solution, by distributing it from a syringe over the surface of the columellar and tarsal muscle regions. The response was visually observed for 10 minutes (n = 3). No visible contraction of columellar or tarsal muscle was observed.

Since ACh is very soluble in water it was initially hypothesized that the ACh solution was unstable (Reynolds 1982). To eliminate this possibility an identical trial was undertaken on whole limpet, *P. oculus*, tarsal muscle with the undiluted ACh (1×10^{-2} M) solution. It caused muscular contraction in the three test individuals which indicated that it had not been hydrolysed in solution and was stable. Lack of activity in *H. midae* could thus indicate that the organism is either insensitive to ACh or has the ability to metabolize externally applied ACh very rapidly or that ACh is not absorbed by the organism.

Once it had been established that the ACh had not been hydrolysed in the solution, and that it stimulated muscular contraction in *P. oculus*, but not in *H. midae*, ACh was excluded from further investigations. The next step was to find an alternative substance to stimulate muscular contraction in *H. midae*. As already mentioned, carbachol is a parasympathomimetic or cholinergic agonist which mimics the actions of ACh on its receptors (Reynolds 1982, Taylor 1991, Watanabe & Katzung 1992). It has also been shown to produce muscle contraction in *M. edulis* (Muneoka *et al.* 1979). The next step was to investigate the effect of carbachol on whole *H. midae* tarsal and columellar muscle. The tarsal and columellar muscle was bathed with an undiluted carbachol solution (1×10^{-3} M) by distributing the solution with a syringe over the surface of these muscles (n = 3). The response of the columellar and tarsal muscle was visually observed for 10 minutes. It caused noticeable contractions of both muscles and was thus used to stimulate *H. midae* muscle contraction in all further investigations.

To determine the effect of the four anaesthetics on the contraction of isolated *H. midae* muscle, it was first necessary to evaluate the carbachol induced contractile response of different sections of abalone tarsal and columellar muscle. Once it had been established

which sections exhibited the strongest contractile response to carbachol, the effect of the four anaesthetics could be tested to evaluate their potential as suitable anaesthetics for abalone.

The apparatus used to determine the contractile responses of different isolated sections of *H. midae* muscle tissue consisted of an isometric transducer coupled to a strain gauge (Bioscience, Palmer & George Washington, U.K.), chart-recorder (Rikadenki), waterbath (Metrohm), aerator and a glass chamber with a tap attached to a clamp stand (Figure 2.1 & 2.2). The chart-recorder displayed the electrical output from the strain gauge which was proportional to the contractile tension developed by the muscle. The chart-recorder deflection was calibrated by suspending a 1 g weight from the transducer and adjusting the displacement thus produced by 97.5%. The glass chamber held the muscle preparation and the bathing solution. Krebs-Henseleit was initially selected as a physiological solution. However, this solution produced spontaneous muscular contraction and could therefore not be used for abalone. Based on the work of Frescura (1990) seawater was consequently selected as a physiological solution. All measurements were made at 18°C.

The following sections of the columellar and tarsal muscle were used to determine which section responds with the strongest and most noticeable contraction to carbachol: dorso-ventral, sagittal and transverse sections of the columellar muscle, and transverse and sagittal sections of the outer ventral surface of the tarsal muscle. The required excisions were made and the preparations mounted individually as follows. Cotton loops were tied securely to both ends of the tissue pieces. One loop was hooked around the aerator inside the organ chamber and the other end was attached to the lever on the transducer. The chamber was filled with 50 ml of pure, filtered seawater (1 micron nylon mesh) and the muscle preparation was left to equilibrate for 2 hours. After two hours had elapsed, 1.0 ml of a 1.0×10^{-3} M carbachol solution was added to the chamber containing 50 ml of seawater to give a final chamber concentration of 2.0×10^{-5} M carbachol.

After evaluating the contractions of the different sections, the sagittal section of the outer, ventral surface of the tarsal muscle was selected as being the most suitable for evaluating the effect of the four anaesthetics on carbachol-induced contractions.

In the actual experiments, fresh muscle preparations were mounted as explained above and left to equilibrate for 2 hours. A single dose of carbachol (1 ml of a 1.0×10^{-3} M solution)

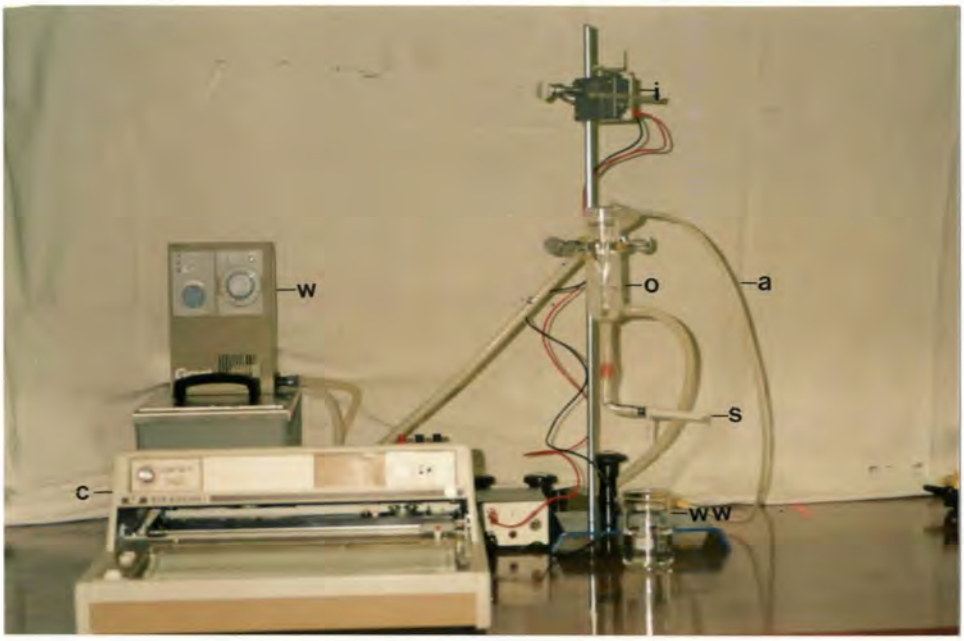


Figure 2.1. Apparatus for investigating the effect of four chemicals on isolated *Haliotis midae* tarsal muscle. a, Aerator; c, chart recorder; i, isometric transducer; o, organ bath; s, stopper; w, waterbath; ww, waste wash.

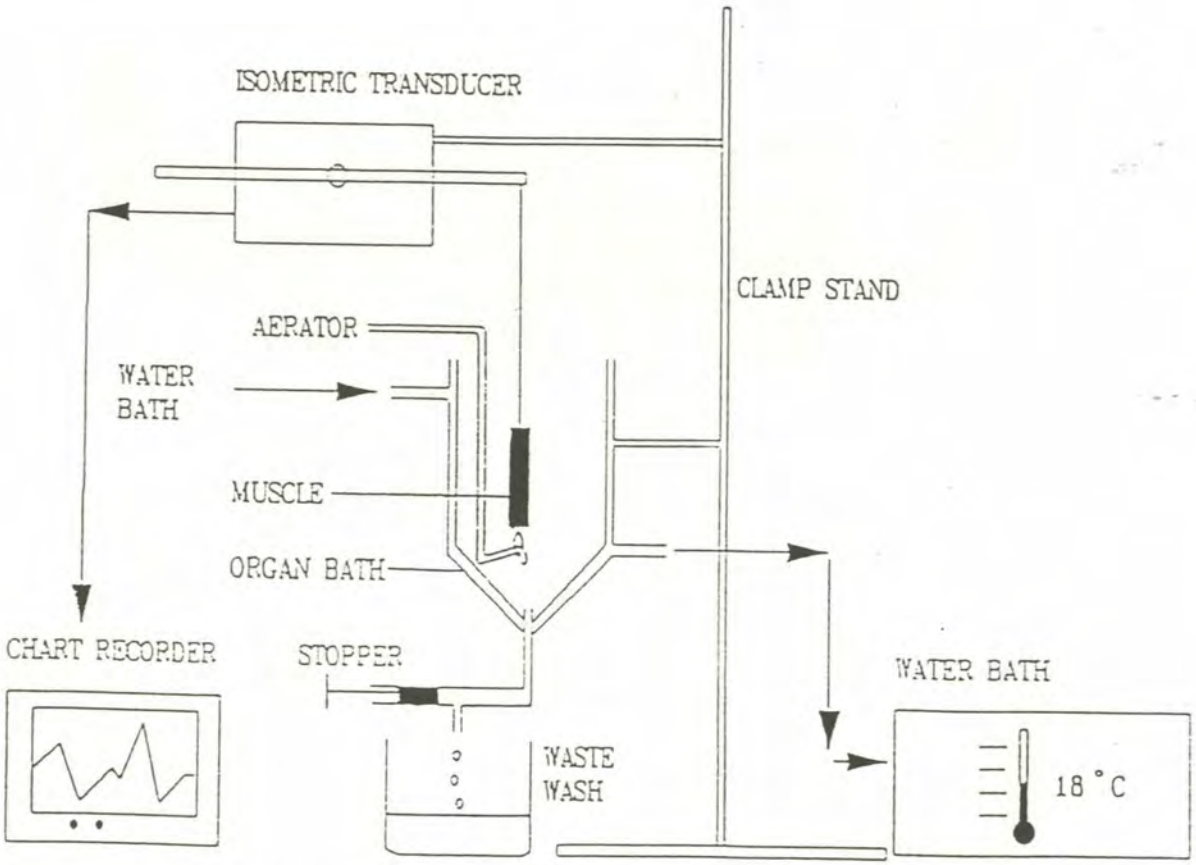


Figure 2.2. Schematic diagram of apparatus for investigating the effect of four chemicals on isolated *Haliotis midae* tarsal muscle.

was then added to the bath containing 50 ml of seawater, which gave a final concentration of 2.0 x 10^{-5} M carbachol. When the muscle contraction reached its peak after 2.5-3 min, the chart-recorder was stopped and the chamber was emptied and refilled with seawater to ensure that no traces of carbachol remained. Once the muscle had relaxed back to baseline, another carbachol contraction was induced in the same manner and the whole process repeated. The two contractions served as a control or base line against which all further responses were compared.

To test the effect of $MgSO_4$ on carbachol-induced contractions, a solution of 5 g $MgSO_4$ dissolved in 100 ml filtered seawater was made up. The chamber was emptied and refilled with 50 ml of the $MgSO_4$ solution to give a final chamber concentration of 2.03 x 10^{-1} M $MgSO_4$. One minute later, a single dose of carbachol (1.0 ml of a 1.0 x 10^{-3} M carbachol solution) was added to the chamber to give a final chamber concentration of 2.0 x 10^{-5} M carbachol and the contraction of the muscle recorded in the presence of $MgSO_4$ for three minutes. After three minutes the chamber was emptied and the tissue rinsed with three changes of pure, filtered seawater to remove all chemicals. The muscle section was given a one hour rest period with three changes of pure, filtered seawater every 20 min. To determine any changes in the contractility of the muscle, two control contractions were then executed with carbachol alone. The same procedure as above was repeated with the muscle preparation using 0.5 ml.100^{-1} ml 2-phenoxyethanol, 2 g.100^{-1} ml procaine hydrochloride and 1 g.100^{-1} ml EDTA (final chamber concentrations of 3.62 x 10^{-2} M, 7.33 x 10^{-2} M and 2.44 x $^{-2}$ M respectively). EDTA is insoluble in seawater and was therefore first dissolved in 20 ml distilled water before making the solution up to 100 ml with filtered seawater. Once again, two control carbachol contractions were executed between each administration after the muscle was allowed a 1 hour rest period with periodic (every 20 min) changes of filtered seawater. If the control carbachol contractions, obtained between the administration of the chemicals, did not differ from the initial control curves, further contractions were compared with the initial contraction curves. However, if they changed, the new deflection was taken as the maximum and further contractions were comparaed with these new controls. The entire procedure described above was replicated with a fresh piece of muscle tissue.

RESULTS

Dorso-ventral, sagittal and transverse sections of the columellar muscle responded poorly to carbachol (Table 2.2). The same was true for transverse sections of the tarsal muscle. The

only excisions that responded with acceptable contractions to carbachol were the sagittal sections of the outer, ventral surface of the tarsal muscle.

Carbachol contractions C_1 to C_6 in the two replicates were all within 5% (viz. 96.85% to 99.40%) and C_7 to C_9 within 5% (viz. 72.88% to 75.60%) of each other, indicating that there was no change in the contractility of the two muscle preparations and that no residual chemicals remained in the tissue (Table 2.3). Although muscle tissue vitality decreased from 99% to 72% (see also Figure 2.3) during the two experimental trials, it was more than adequate to test the inhibitory effect of the various chemicals.

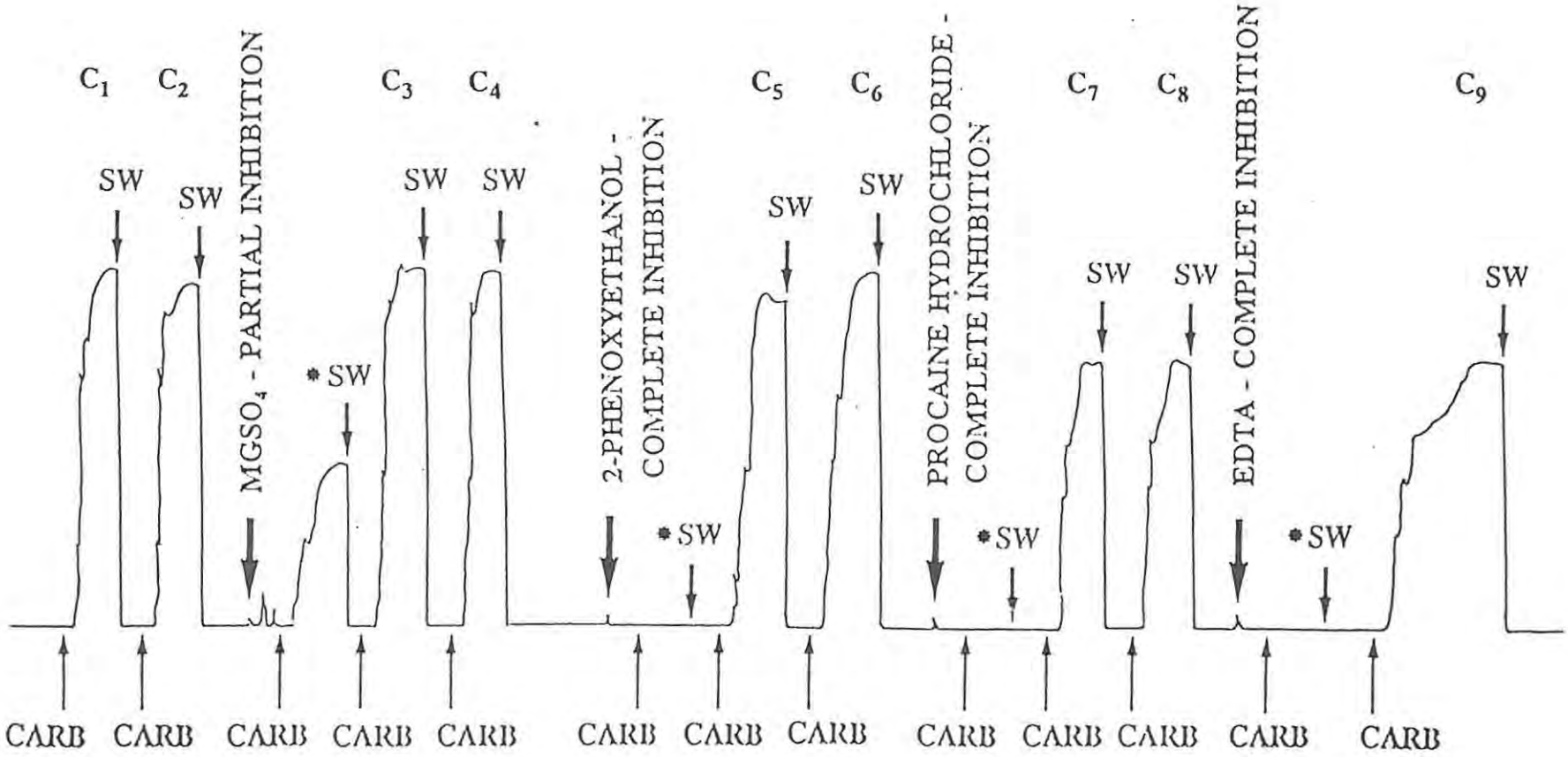
Table 2.2. The response of different sections of *Haliotis midae* columellar and tarsal muscle to carbachol.

Muscle	Section	Observed response
Columellar	Dorso-ventral	Weak contraction
Columellar	Sagittal	Weak contraction
Columellar	Transverse	Weak contraction
Tarsal	Sagittal	Strong contraction
Tarsal	Transverse	Weak contraction

Table 2.3. The mean percentage peak heights of the control carbachol induced contractions obtained in the two replicates.

Carbachol induced contraction	Mean percentage height of response curve
C_1	99.40±0.61
C_2	97.42±2.58
C_3	98.79±1.29
C_4	93.40±2.49
C_5	96.31±2.50
C_6	96.85±1.09
C_7	75.60±1.37
C_8	74.00±1.27
C_9	72.88±1.36

Figure 2.3. Inhibition of carbachol-induced contractions of isolated *Haliotis midae* tarsal muscle tissue in the presence of $MgSO_4$, 2-phenoxyethanol, procaine hydrochloride and EDTA. (SW = seawater, CARB = carbachol, C = control carbachol response-curve). Each tissue rinse (SW) included three changes of pure, filtered seawater every 5 min except for those indicated with an asterisk which included three changes of pure, filtered seawater every 20 min.



All four chemicals inhibited the contractile effect of carbachol on isolated sagittal sections of the outer, ventral surface of the tarsal muscle. Treatment with 2-phenoxyethanol, procaine hydrochloride and EDTA completely eliminated the muscular contraction produced by carbachol. However, when carbachol was added in the presence of $MgSO_4$, the inhibitory effect was only 50% in relation to the carbachol induced contractions. The possible reasons for this are considered in the discussion.

DISCUSSION

The observation that ACh did not stimulate contraction in whole abalone muscle is either a consequence of insufficient absorption into the tissue through the skin, or hydrolysis in the tissue and thus rendered inactive. It is known that the poor absorption and distribution of ACh is principally a factor of its low lipid solubility (Watanabe & Katzung 1992). In mammals ACh is hydrolysed by two cholinesterases and large amounts must be infused intravenously to achieve levels which are high enough to produce detectable effects in mammalian muscle (Bowman & Rand 1980, Lefkowitz *et al.* 1991, Katzung 1992). The first type of cholinesterase is acetylcholinesterase which is present in large quantities in the synapses. The second type is butyrocholinesterase or pseudocholinesterase, found in blood plasma, skin, liver and other tissue. The poor absorption rate and/or rapid hydrolysis of ACh as described above, possibly provides an explanation why it did not stimulate contraction in whole abalone muscle. However, given that ACh did cause contraction of limpet muscle would suggest that abalone could have higher levels of cholinesterase than limpets. Carbachol, on the other hand, is completely resistant to hydrolysis by cholinesterase and has a longer duration of action than ACh (Taylor 1991, Watanabe & Katzung 1992). This would support the suggestion that higher cholinesterase levels are present in abalone than in limpet, and that this could be the principle reason why ACh did not effect contractions of abalone muscle.

The only muscle tissue sections that visibly responded to carbachol were sagittal sections of the outer, ventral surface of the tarsal muscle. The probable reason for this is the higher prevalence of sarcolemmal cisternae in this muscle which allows for rapid relaxation and increased flexibility (Voltzow 1990), coupled with the smaller thick filament and muscle cell diameter which increases the speed of contraction (Voltzow 1990, see also Chapter 6).

$MgSO_4$, 2-phenoxyethanol, procaine hydrochloride and EDTA inhibited muscle contraction in isolated *H. midae* tarsal muscle tissue. Procaine hydrochloride impairs nerve transmission by acting both pre- and postjunctionally (Bowman & Rand 1980). It is very likely that 2-phenoxyethanol also exerts its effect in this manner. EDTA labilizes excitable membranes by decreasing the availabilty of calcium ions. These three chemicals therefore caused "flaccid" paralysis of the muscle. $MgSO_4$, on the other hand, only interferes with ACh release from the nerve terminal (Bowman & Rand 1980) and therefore simply effected partial relaxation of the muscle, manifested by the partial inhibition of muscle contraction. Overall, the results showed that each of these chemicals has potential for abalone anaesthesia and therefore warranted further evaluation.

CHAPTER 3

THE SIZE RELATED EFFECTS OF MAGNESIUM SULPHATE, 2-PHENOXYETHANOL, PROCAINE HYDROCHLORIDE, ETHYLENEDIAMINE TETRA-ACETIC ACID, BENZOCAINE AND CARBON DIOXIDE ANAESTHESIA

INTRODUCTION

In vitro investigation into the effect of magnesium sulphate ($MgSO_4$), 2-phenoxyethanol, procaine hydrochloride and ethylenediamine tetra-acetic acid (EDTA) on isolated *Haliotis midae* tarsal muscle indicated that all four chemicals have potential as abalone anaesthetics (see Chapter 2). This information provided a baseline for establishing the *in vivo* anaesthetic potential of these chemicals on different size classes of *H. midae*. Two additional anaesthetics, carbon dioxide (CO_2) and benzocaine, were also included in this evaluation as they are also known to induce anaesthesia in abalone (Sugiyama & Tanaka 1982, Hahn 1989d, Tong *et al.* 1992, Anonymous 1994).

The efficacy of an anaesthetic is subject to its ability to meet the requirements of the abalone farmer. It was necessary therefore to first establish guideline criteria for the evaluation of efficacy. It is well known that mechanical removal of abalone from any substratum can damage the animals, resulting in mortality. The fundamental criteria were to discover an anaesthetic medium, and to develop a protocol, that would result in mortality-free anaesthesia. The second consideration was the rate of anaesthesia. Consultation with some members of the industry revealed that an acceptable anaesthesia rate for industrial purposes would be between 5-20 minutes (C. Claydon, Sea Plant Products, Hermanus, pers. comm.). It was also argued by industry that a shorter exposure time would be advantageous as presumably there is less time for the build-up of residues in the tissue. This is not only important during the culturing process, but also immediately prior to exportation as the animals would have to be anaesthetized for grading purposes before being shipped to their final destination.

The development of a size-related dosage table was the primary objective of this investigation. This would provide the abalone farmer with important information such as the time required for specific concentrations to effect anaesthesia and the concentrations required for different size classes of abalone. Effective concentration also affects costs which is an important consideration in the commercial situation.

MATERIALS AND METHODS

Three size classes (5-15, 20-50 and 60-90 mm shell length (SL)) were selected for this study. The small animals (5-15 mm SL) were obtained from the Sea Plant Products hatchery in Hermanus, while larger animals (20-50 and 60-90 mm SL) were collected along the Eastern Cape Coast. Prior to the initiation of the experiments, the animals were acclimated to laboratory conditions for a period of one month. During the acclimation period the animals were kept at a temperature of 18°C and a salinity of 35 ppt. All experiments were conducted at this temperature and salinity.

The effect of a range of concentrations of $MgSO_4$, 2-phenoxyethanol, procaine hydrochloride, EDTA and benzocaine as well as a range of flow rates of a CO_2 : O_2 gas mixture were evaluated. Initial concentrations for each size class were selected on a trial and error basis. The final concentrations tested were based around the initial test concentrations, depending on the rate of anaesthesia. The gas mixture that was selected for this study (11.3% CO_2 : 88.7% O_2) was based on that used in fish (Itazawa 1983, Yoshikawa *et al.* 1988a, 1988b, Yokoyama *et al.* 1989).

The following replicate trials were undertaken: four replicates for each concentration of $MgSO_4$, EDTA, and 2-phenoxyethanol for each of the three size classes; four replicates for each concentration of benzocaine for the two smaller size classes (5-15 and 20-50 mm SL) and three replicates for each concentration of benzocaine for the largest size class (60-90 mm SL); three replicates for each flow rate of CO_2 for each of the three size classes and four replicates for each concentration of procaine hydrochloride for the two smaller size classes (5-15 and 20-50 mm SL). The trials were undertaken in plastic buckets containing 5 litres of continuously aerated seawater. Ten animals were placed in each bucket and allowed to attach to the sides. To obtain the required dose ranges, the chemicals were either administered on a weight basis ($MgSO_4$ and procaine hydrochloride) or on a volumetric basis (2-phenoxyethanol, EDTA and benzocaine). EDTA is insoluble in seawater and was therefore first dissolved in distilled water (1:20) prior to mixing it with seawater. Benzocaine was dissolved in 95% ethanol (1:8) prior to mixing it with seawater (Reynolds 1982, Tong *et al.* 1992). Once all the animals were firmly attached to the substratum, the seawater was poured out and replaced with the anaesthetic solution.

Anaesthesia with CO_2 was induced by bubbling the gas, via a diffuser, through the water. The flow rate through the experimental chamber was controlled with a Messer Griesheim gas-flow regulator. O_2 and pH levels in the seawater were measured before commencing each run and once anaesthesia had been complete. The time taken for each animal to be completely anaesthetized was noted to the nearest 1 ± 0.5 sec. Complete anaesthesia was defined as the inability of the abalone to adhere onto the side or bottom of the experimental chamber. To terminate anaesthesia, the animals were transferred to a bucket containing fresh, aerated seawater and placed upside down on the bottom of the container. The time taken for each animal to recover was noted. Recovery was defined to be complete when the abalone turned right side up.

As the disodium salt of EDTA was used a separate experiment was conducted to evaluate the effect of different concentrations of EDTA on the salinity of the seawater. The concentrations of EDTA used to anaesthetize the animals were dissolved in distilled water and made up to the required volume with seawater. The salinity of the seawater was measured before and after EDTA had been added. Three replicates were undertaken for each concentration.

Statistical analysis

To test the effect of concentration, or flow rate in the case of CO_2 on anaesthesia and recovery rate, data were subjected to One-Way Analysis of Variance, with the main effects being concentration or flow rate. Means were compared using Tukey's Multiple Range Test at 5% error probability. Homogeneity of variances were tested with Bartlett's Test. When required, the natural logarithm transformation was used to stabilise the variances. Simple regression procedure was used to obtain the best model to predict the anaesthesia rate for the three most suitable chemicals as a function of concentration or flow rate. The model with the highest T-value for the slope, the highest F-ratio and most random residual pattern was chosen to be the best fit. When the models were found to be unsuitable for predicting anaesthesia rate as a function of concentration or flow rate, the data were subjected to the Kruskal-Wallis One-Way Analysis by Ranks procedure. This procedure was used to analyze whether the distribution of anaesthesia rate varies with concentration.

The pH, O_2 levels and salinities measured during the CO_2 and EDTA trials, were subjected to One-Way Analysis of Variance, with the main effects being flow rate (CO_2) and concentration (EDTA). Means were compared using Tukey's Multiple Range Test at 5%

error probability. Homogeneity of variances were tested with Bartlett's Test (Zar 1984).

RESULTS

Exposure to different concentrations of $MgSO_4$, 2-phenoxyethanol, procaine hydrochloride, EDTA and benzocaine, and continuous bubbling of the CO_2 gas mixture at different flow rates all resulted in complete anaesthesia in all three abalone size classes. The mean time to effect complete anaesthesia, mean recovery rate, percentage recovery and percentage survival at each of the concentrations and flow rates are shown in Tables 3.1 to 3.3. One-Way Analysis of Variance showed that there was an inverse relationship between concentration or flow rate and rate of anaesthesia; the higher the concentration the more rapid the rate of anaesthesia. Statistical differences between mean anaesthesia rates for different concentrations or flow rates are shown in Tables 3.1, 3.2 and 3.3 using different superscripts ($p < 0.05$). The only instance in which there was not an inverse relationship between effective concentration and rate of anaesthesia was for the smallest size class subjected to different 2-phenoxyethanol and $MgSO_4$ concentrations (see Table 3.1). There is no pharmacological explanation for this phenomenon.

Abalone size was also positively correlated with effective concentration or flow rate; higher concentrations were required for larger animals. Statistical differences in recovery rates at different concentrations are shown in Tables 3.1, 3.2 and 3.3. There were no apparent trends in the recovery rates within the size classes. However, higher concentrations and flow rates were required for larger animals which resulted in longer recovery times. All the animals anaesthetized with $MgSO_4$, 2-phenoxyethanol and CO_2 recovered once they were placed in fresh seawater. Half of the small abalone (5-15 mm SL) treated with procaine hydrochloride at a concentration of 0.5 g.100 ml^{-1} did not recover from anaesthesia. All the medium size animals (20-50 mm SL) treated with the same concentration of procaine hydrochloride recovered, but died after 48 hours. Because of the high mortalities recorded at a concentration of 0.5% in the two smaller size classes, no further experiments were undertaken with procaine hydrochloride. Post-recovery mortalities were also recorded in the EDTA treatments. All the animals in the 60-90 mm SL groups treated at a concentration of 5.0 g.100 ml^{-1} died after 48 hours (Table 3.2). Mortalities were also recorded in the benzocaine treatments, during and after anaesthesia (Table 3.3).

Table 3.1. The mean rate of anaesthesia, recovery, percentage recovery and percentage survival of three size classes of *Haliotis midae* after exposure to $MgSO_4$ and 2-phenoxyethanol. Standard deviations of the means are also presented. Different superscripts indicate significant differences in mean anaesthesia and recovery rates at $p<0.05$ (Note: although the same superscripts were used to denote statistical differences or similarities, the anaesthesia and recovery rates were analyzed separately and should be interpreted as such).

$MgSO_4$					
Size (mm SL)	**Conc. g.100 ml^{-1}**	**Mean rate of anaesthesia (min)**	**Mean recovery rate (min)**	**Percentage recovery**	**Percentage survival**
5-15	2	25.0±15.8^{a}	8.5±3.9^{b}	100	100
	3	4.9±4.9^{b}	14.5±5.7^{a}	100	100
	4	2.0±2.2^{bc}	9.2±4.9^{b}	100	100
	6	* 0.8±1.2^{c}	6.1±3.6^{c}	100	100
	8	*2.0±2.1^{bc}	8.9±4.1^{b}	100	100
	10	0.3±0.3^{d}	2.9±2.0^{d}	100	100
20-50	6	18.5±8.8^{e}	26.5±4.0^{e}	100	100
	8	10.9±7.2^{f}	22.9±7.9^{ef}	100	100
	10	8.0±5.3^{f}	21.8±8.2^{ef}	100	100
	12	6.6±4.5^{fg}	18.0±8.5^{f}	100	100
	14	5.2±5.0^{gh}	24.0±10.2^{e}	100	100
	16	3.8±2.3^{gh}	11.8±4.7^{g}	100	100
	18	2.4±2.0^{h}	8.0±3.3^{h}	100	100
60-90	14	47.8±19.3^{i}	134.8±18.6^{i}	100	100
	16	38.7±9.6^{i}	77.2±27.4^{k}	100	100
	18	30.3±15.2^{j}	85.3±14.6^{k}	100	100
	20	25.0±13.2^{j}	112.5±28.5^{j}	100	100
	22	9.3±4.9^{k}	35.2±20.2^{l}	100	100
	24	3.8±2.7^{l}	22.1±13.0^{i}	100	100
2-phenoxyethanol	**ml.100 ml^{-1}**				
5-15	0.05	* 0.8±0.7^{m}	2.6±0.8^{m}	100	100
	0.1	* 1.6±0.7^{n}	4.8±2.7^{n}	100	100
	0.15	0.8±0.4^{o}	3.1±2.1^{m}	100	100
	0.2	0.2±0.1^{p}	2.4±1.5^{m}	100	100
20-50	0.05	4.1±1.4^{q}	7.6±3.0^{o}	100	100
	0.1	4.8±2.2^{q}	13.6±6.8^{pq}	100	100
	0.2	1.8±1.1^{r}	13.2±8.1^{op}	100	100
	0.3	1.1±0.9^{s}	23.6±13.1^{r}	100	100
	0.4	0.4±0.1^{t}	23.1±9.5^{r}	100	100
	0.5	0.4±0.5^{t}	21.1±12.3^{qr}	100	100
60-90	0.1	7.0±3.7^{u}	17.2±6.8^{s}	100	100
	0.3	2.9±2.3^{v}	46.0±24.6^{t}	100	100
	0.5	1.1±1.0^{w}	39.9±14.8^{t}	100	100
	0.7	0.8±0.5^{w}	42.9±23.9^{t}	100	100

* Deviations from inverse relationship between concentration and rate of anaesthesia.

Table 3.2. The mean rate of anaesthesia, recovery, percentage recovery and percentage survival of three size classes of *Haliotis midae* after exposure to EDTA and two size classes after exposure to procaine hydrochloride. Standard deviations of the means are also presented. Different superscripts indicate significant differences in mean anaesthesia and recovery rates at $p<0.05$ (Note: although the same superscripts were used to denote statistical differences or similarities, the anaesthesia and recovery rates were analyzed separately and should be interpreted as such).

EDTA					
Size (mm SL)	**Conc. g.100 ml^{-1}**	**Mean rate of anaesthesia (min)**	**Mean recovery rate (min)**	**Percentage recovery**	**Percentage survival**
5-15	0.1	2.3±2.6^{a}	5.0±3.4^{a}	100	100
	0.3	0.7±0.5^{b}	1.6±1.2^{c}	100	100
	0.5	0.3±0.2^{c}	0.9±0.4^{b}	100	100
	1.0	0.2±0.1^{c}	3.0±1.3^{d}	100	100
20-50	0.5	5.8±4.2^{d}	5.8±2.1^{e}	100	100
	1.0	3.5±2.5^{d}	10.0±3.8^{f}	100	100
	2.0	2.1±1.5^{e}	8.9±3.5^{f}	100	100
	3.0	0.7±0.7^{f}	10.4±3.6^{f}	100	100
60-90	1.0	7.1±4.4^{g}	14.7±4.4^{g}	100	100
	3.0	4.9±2.6^{g}	22.6±6.3^{h}	100	100
	5.0	1.7±1.2^{h}	29.4±11.0^{i}	100	0
Procaine hydrochloride	**g.100 ml^{-1}**				
5-15	0.5	3.2±4.3	26.2±20.8	50	0
20-50	0.5	2.2±1.8	7.5±2.8	100	0

Statistical differences in pH and O_2 levels were recorded during the CO_2 anaesthesia trials (Table 3.4). At each of the flow rates, except at 6 l.min^{-1} for the 5-15 mm SL *H. midae*, a mean pH change of 2±0.05 and a mean O_2 level change of 25±0.8 mg.l^{-1} was noted. The mean pH and O_2 level change at the 6 l.min^{-1} flow rate for the 5-15 mm SL *H. midae* was 1.71 and 17.4 mg.l^{-1} respectively.

The use of the disodium salt of EDTA resulted in an increase in salinity of the seawater at all concentrations (Table 3.5).

The predicted anaesthesia rate as a function of concentration or flow rate for the three most suitable anaesthetics ($MgSO_4$, 2-phenoxyethanol and CO_2), are shown in Figures 3.1 to 3.8. Anaesthesia rate could not be predicted as a function of concentration for the smallest size

class (5-15 mm SL) anaesthetized with 2-phenoxyethanol. However, Kruskal-Wallis analysis showed that there were significant differences between the mean anaesthesia rates ($p < 0.05$). These statistical differences in mean anaesthesia rates are confirmed by One-Way Analysis of Variance (Tables 3.1 and 3.3).

Table 3.3. The mean rate of anaesthesia, recovery, percentage recovery and percentage survival of three size classes of *Haliotis midae* after exposure to benzocaine and a CO_2 : O_2 gas mixture (11.3% : 88.7%). Standard deviations of the means are also presented. Different superscripts indicate significant differences in mean anaesthesia and recovery rates at $p < 0.05$ (Note: although the same superscripts were used to denote statistical differences or similarities, the anaesthesia and recovery rates were analyzed separately and should be interpreted as such).

Benzocaine					
Size (mm SL)	**Conc. g.100 ml-1**	**Mean rate of anaesthesia (min)**	**Mean recovery rate (min)**	**Percentage recovery**	**Percentage survival**
5-15	0.0003	1.1 ± 0.8^{a}	1.4 ± 0.7^{b}	100	100
	0.0005	1.0 ± 0.6^{a}	0.6 ± 0.4^{a}	100	100
	0.001	0.9 ± 0.5^{a}	1.4 ± 0.6^{b}	100	100
	0.003	0.6 ± 0.3^{a}	1.6 ± 0.7^{b}	100	100
	0.005	0.2 ± 0.1^{b}	1.7 ± 0.7^{b}	100	100
20-50	0.005	2.4 ± 1.9^{c}	5.2 ± 2.1^{c}	100	100
	0.01	2.1 ± 1.6^{cd}	6.4 ± 3.2^{c}	100	100
	0.02	1.6 ± 1.3^{cde}	9.9 ± 7.3^{c}	100	100
	0.04	1.0 ± 0.9^{de}	8.1 ± 5.4^{d}	100	80
	0.06	0.8 ± 0.6^{e}	7.4 ± 2.9^{d}	100	73
60-90	0.05	9.2 ± 3.1^{f}	84.0 ± 20.6^{f}	100	87
	0.1	7.7 ± 2.1^{f}	$>4000^{g}$	90	27
	0.2	3.7 ± 2.4^{g}	315.5 ± 64.8^{h}	100	100
CO_2	**Flow rate l.min^{-1}**				
5-15	3	5.9 ± 4.4^{h}	3.0 ± 2.8^{i}	100	100
	4	3.1 ± 2.3^{h}	2.7 ± 2.6^{i}	100	100
	5	1.6 ± 1.3^{i}	1.8 ± 1.7^{i}	100	100
	6	0.6 ± 0.6^{j}	1.1 ± 0.6^{i}	100	100
20-50	2	10.2 ± 8.7^{k}	3.8 ± 1.9^{j}	100	100
	3	5.6 ± 4.1^{kl}	4.0 ± 2.0^{j}	100	100
	4	2.7 ± 1.7^{l}	0.6 ± 0.3^{k}	100	100
	5	1.2 ± 1.0^{m}	0.8 ± 0.5^{k}	100	100
	6	1.1 ± 0.9^{m}	0.9 ± 0.5^{k}	100	100
	7	0.8 ± 0.7^{m}	0.7 ± 0.4^{k}	100	100
60-90	6	12.6 ± 9.6^{n}	5.7 ± 3.5^{l}	100	100
	8	8.5 ± 3.6^{n}	2.8 ± 2.7^{m}	100	100
	10	4.5 ± 2.8^{o}	2.6 ± 1.6^{m}	100	100
	12	2.6 ± 2.1^{o}	1.9 ± 1.3^{m}	100	100

Table 3.4. The mean pH and O_2 levels measured in the test tanks before and after anaesthesia of three size classes of three size classes of *Haliotis midae* with a CO_2 : O_2 gas mixture (11.3% : 88.7%). Different superscripts indicate significant differences in mean pH and O_2 level changes at $p<0.05$.

Size (mm SL)	Flow rate ($l.min^{-1}$)	Mean total exposure time to the gas mixture (min)	Mean O_2 concentration ($mg.l^{-1}$)			Mean pH		
			Start of run	End of run	Change	Start of run	End of run	Change
5-15	3	13.62	8.67	34.00^{a}	25.33^{c}	7.91	5.91^{ef}	2.01^{h}
	4	7.06	8.67	33.97^{a}	25.30^{c}	7.88	5.93^{e}	1.95^{h}
	5	3.61	8.63	33.77^{a}	25.13^{c}	7.89	5.90^{ef}	1.99^{h}
	6	1.69	8.47	25.87^{b}	17.40^{d}	7.92	6.21^{g}	1.71^{i}
20-50	2	23.76	8.97	34.17^{a}	25.20^{c}	7.90	5.86^{ef}	2.04^{h}
	3	13.15	8.73	34.00^{a}	25.27^{c}	7.89	5.90^{ef}	1.99^{h}
	4	5.31	8.83	34.07^{a}	25.23^{c}	7.86	5.86^{ef}	2.00^{h}
	5	3.03	8.80	33.90^{a}	25.10^{c}	7.87	5.88^{ef}	1.99^{h}
	6	2.44	8.67	33.73^{a}	25.07^{c}	7.89	5.90^{ef}	1.99^{h}
	7	1.98	8.77	33.03^{a}	24.27^{c}	7.88	5.92^{ef}	1.96^{h}
60-90	6	30.92	8.77	34.13^{a}	25.37^{c}	7.86	5.88^{ef}	1.98^{h}
	8	14.08	8.50	34.13^{a}	25.63^{c}	7.92	5.92^{ef}	2.00^{h}
	10	8.79	8.87	34.40^{a}	25.53^{c}	7.85	5.83^{f}	2.02^{h}
	12	6.58	8.53	33.93^{a}	25.40^{c}	7.91	5.88^{ef}	2.03^{h}

Table 3.5. Salinity changes in seawater after the addition of different concentrations of EDTA. Different superscripts indicate significant differences in mean salinity level changes at $p<0.05$.

Concentration of EDTA ($g.100\ ml^{-1}$ seawater)	Mean salinity of seawater at end of run (ppt)	Mean salinity change of seawater (ppt)
0.1	35.5^{a}	0.5^{f}
0.3	36.0^{ab}	1.0^{fg}
0.5	36.5^{ab}	1.5^{fg}
1.0	37.0^{bc}	2.0^{gh}
2.0	38.0^{cd}	3.0^{hi}
3.0	39.0^{d}	4.0^{i}
5.0	42.0^{e}	7.0^{j}

Salinity at the start of each run was 35.0 ppt.

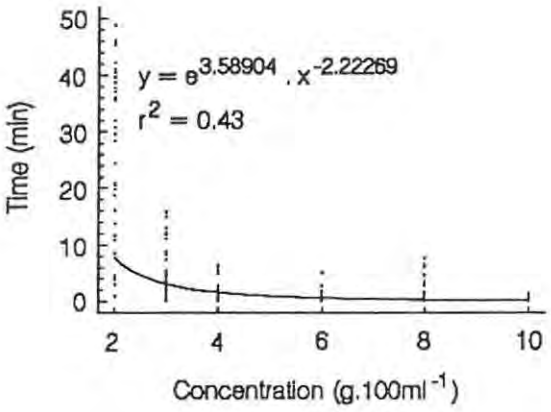


Figure 3.1. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different concentrations of $MgSO_4$.

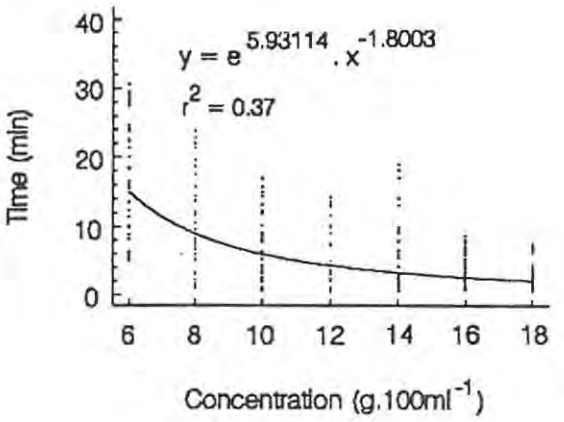


Figure 3.2. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different concentrations of $MgSO_4$.

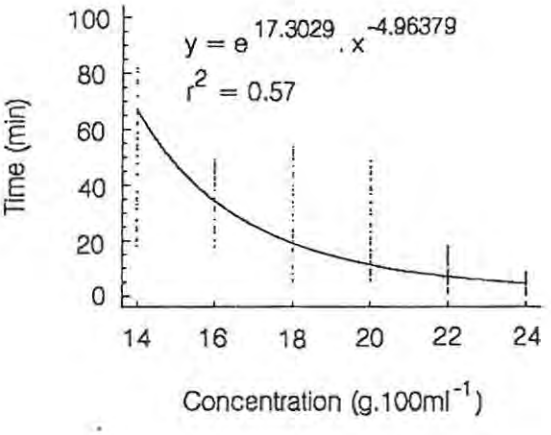


Figure 3.3. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different concentrations of $MgSO_4$.

DISCUSSION

EDTA, procaine hydrochloride and benzocaine failed to meet the efficacy criteria as abalone anaesthetics in terms of recovery and survival rate. At a concentration of 5.0 g.100 ml^{-1}, EDTA effectively caused anaesthesia in the largest size class, but all the animals died 48 hours after recovery. It was hypothesized that the cause of death might not have been related to the anaesthetic, but could have been a consequence of the animals being exposed to a rapid

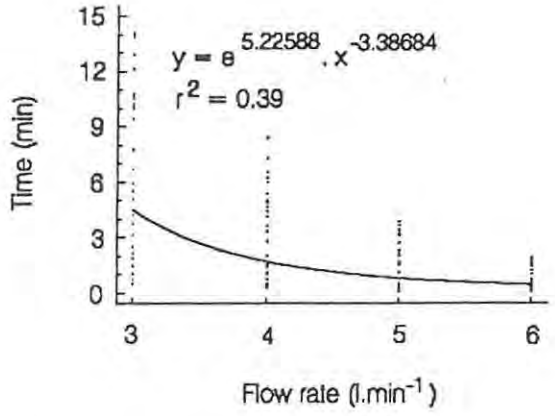


Figure 3.4. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture.

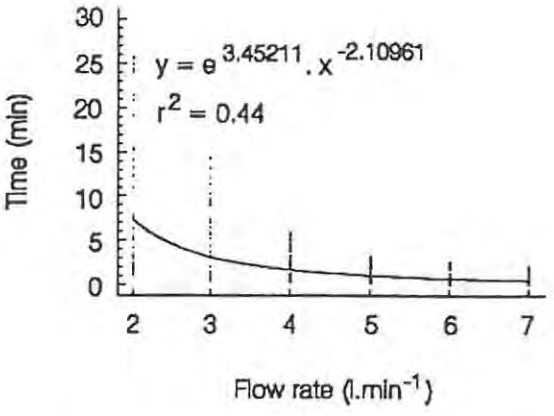


Figure 3.5. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture.

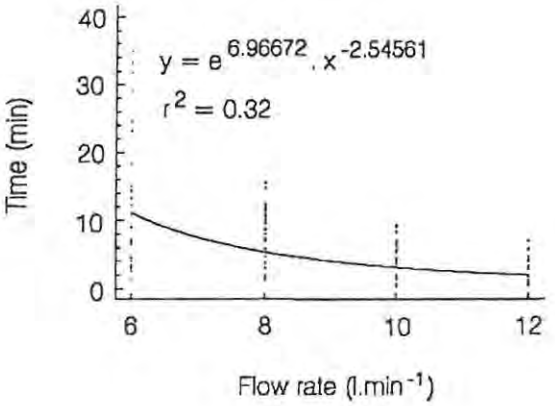


Figure 3.6. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture.

salinity change (Hahn 1989d). At a concentration of 5.0 g.100 ml^{-1} EDTA, the disodium salt effected a 7 ppt increase in salinity to which the animals were instantaneously exposed. This may have been the cause of death 48 hours later. Another disadvantage of EDTA is that it is insoluble in seawater and needs to be dissolved in distilled water (1:20) and then made up to the required volume with seawater. EDTA is therefore impractical and unsuitable for commercial application.

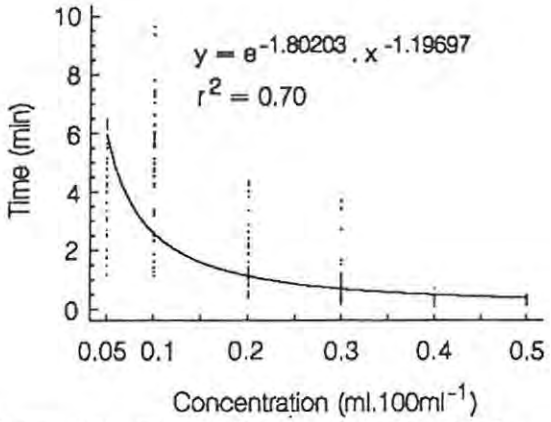


Figure 3.7. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different concentrations of 2-phenoxyethanol.

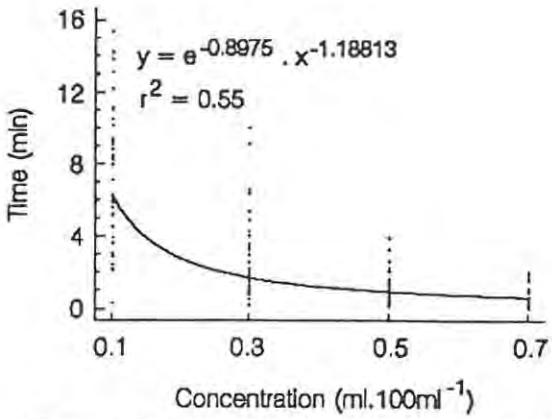


Figure 3.8. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different concentrations of 2-phenoxyethanol.

Procaine hydrochloride also resulted in mortalities in the two smallest size classes and was therefore also regarded to be unsafe for commercial application. Mortalities were also recorded for benzocaine during and after anaesthesia. Recovery times after benzocaine anaesthesia were much too long in some instances. For example, the recovery times for 60-90 mm SL animals anaesthetized with 0.2 and 0.1 g.100 ml^{-1} benzocaine were in excess of 300 and 4000 min, respectively. These recovery times are not acceptable in a commercial situation. While no mortalities were observed during and after 0.2 g.100 ml^{-1} benzocaine anaesthesia, mortalities of 13% and 73% were observed for the 0.05 and 0.1 g.100 ml^{-1} benzocaine treatments, respectively. These mortalities confirm the fears of the industry regarding the efficiency and reliability of benzocaine as an anaesthetic for abalone.

The recovery rates after CO_2 (11.3 % CO_2 : 88.7% O_2) anaesthesia at a flow rate of 2 l/min were compared to the recovery rates reported by Sugiyama and Tanaka (1982) for juvenile *Nordotis discus* (8.3-29.8 mm SL). The recovery rates observed in this study were faster than the recovery rates reported by Sugiyama and Tanaka (1982) for four CO_2 gas mixtures (25, 50, 75 and 100 % CO_2) bubbled through 5 l of seawater at a flow rate of 2 l/min. The higher CO_2 levels in the gas mixtures used by Sugiyama and Tanaka (1982) would have effected higher partial pressures of CO_2 in the water, resulting in a drop in pH (Spotte 1979).

Intracellular pH is actively regulated and is very sensitive to changes in the external partial pressure of CO_2 (Izutsu 1972, Saborowski *et al.* 1973, Khuri *et al.* 1974). An increase in

external CO_2 concentration has been found to result in a large and rapid decrease in internal pH (Izutsu 1972, Thomas 1974). Maintenance of intracellular pH is of major importance, since it provides suitable conditions for the activity of cellular multi-enzyme systems (Malan *et al.* 1976). Enzymes exert their activity over a limited range of pH and very often a definitive optimum pH is required. Moreover, enzymes have pH-sensitive ionising groups which contribute to the pattern of electrical charge over the enzyme surface, and these regulate the extent to which the enzyme interacts with any substance whether substrate, activator, coenzyme or inhibitor. Williams *et al.* (1971a, 1971b) found a strong correlation between decreased intracellular pH, transmembrane resting potential and ion (H^+ and Na^+) transport. Decreased intracellular pH influences Na^+-K^+ activated ATPase which in turn plays a major role in muscle contraction (Martin 1987). The rate of Ca^{2+} uptake into mitochondria is also affected by pH (Martin 1987). A decrease in extramitochondrial pH will decrease the rate of calcium uptake and in excitable cells such as muscle, calcium influx into the cell is very important in the regulation of cell function. Since intracellular pH is very sensitive to changes in the partial pressure of CO_2, the differences in recovery times reported by Sugiyama and Tanaka (1982) and those reported here were most probably a consequence of the extent of intracellular pH change. The intracellular pH in the animals anaesthetized with the 25, 50, 75 and 100 % CO_2 gas mixtures by Sugiyama & Tanaka (1982) was most likely decreased to a greater extent than in animals anaesthetized with the 11 % CO_2 gas mixture in this study. This probably affected the enzyme systems, and therefore the transmembranal movement of ions required for muscle contraction to a greater extent in the animals anaesthetized with the higher concentrations of CO_2. Thus, when animals are placed in fresh seawater after low level CO_2 anaesthesia, the enzyme activity (and therefore muscle activity) is expected to recover more rapidly due to the faster normalization of intracellular pH levels. This explains the differences between the recovery rates reported by Sugiyama & Tanaka (1982) and those found in this study, as well as the inverse relationship between anaesthesia rate and flow rate of the CO_2 gas mixture in this study.

Only three of the six chemicals tested, $MgSO_4$, 2-phenoxyethanol and the CO_2 gas mixture, met the criteria of an effective abalone anaesthetic as they induced rapid and mortality-free anaesthesia in all three size classes of *H. midae*. $MgSO_4$, commonly known as epsom salts, is freely available over-the-counter as a mild laxative for human medicinal use. A minor disadvantage of $MgSO_4$ is that relatively large concentrations were required for anaesthesia. 2-Phenoxyethanol has been used widely in fish (Ross & Ross 1984, Marking & Meyer 1985,

Gilderhus & Marking 1987, Yamamitsu & Itazawa 1988, Iwama *et al.* 1989, Mattson & Riple 1989, Teo *et al.* 1989, Teo & Chen 1993). It is not classified as a hazardous substance, although, its MSDS (Material Safety Data Sheet) from Sigma Chemicals Corporation (U.S.A./Canada) states that it is harmful if swallowed and can cause ocular and respiratory irritation (M.A. Carelaru, Sigma Chemicals Corporation, St. Louis, U.S.A., pers. comm. and T. Hall, Whitehead Scientific Laboratories, Cape Town, pers. comm.). Safety precautions also require the user to wear protective clothing. CO_2 has been used as an anaesthetic in almost every animal phylum (Ross & Ross 1984). It has also been shown to be an efficient and cost-effective anaesthetic for fish, as well as for other aquatic organisms, including abalone (Sugiyama & Tanaka 1982). Other advantages of CO_2 is that it leaves no residues, requires no depuration period and requires no registration, since its use in food fish is already allowed under the present GRAS (generally regarded as safe) declaration by the U.S.A. Food and Drug Administration (FDA) (Gilderhus & Marking 1987).

In conclusion, the data obtained in this study provides the commercial farmer with important information. The dosage tables and the predicted concentrations for $MgSO_4$ and 2-phenoxyethanol or flow rates for CO_2 (see Figures 3.1 to 3.8) can be used as guidelines for the effective anaesthesia of different sized animals. The three most suitable chemicals therefore warranted further consideration as anaesthetics for *H. midae*. The effect of temperature on anaesthesia rate as well as the long term physiological effects of regular anaesthesia will be dealt with in the next chapters.

CHAPTER 4

THE EFFECT OF TEMPERATURE ON THE EFFICACY OF MAGNESIUM SULPHATE AND CARBON DIOXIDE ANAESTHESIA IN *HALIOTIS MIDAE*

INTRODUCTION

The efficacy of anaesthetics can vary widely with water temperature (Gilderhus & Marking 1987). The general tendency is for anaesthesia to be effected quicker at higher temperatures. Gilderhus (1989) showed that the efficacy of benzocaine for salmonid fishes (*Oncorhynchus tshawytscha* and *O. mykiss*) was related to water temperature and fish size; the concentrations of benzocaine required for effective anaesthesia of these salmonid fish was the highest at the lowest water temperature and for the largest fish. Similarly, concentrations of benzocaine required for anaesthesia of *Prochilodus lineatus* and *Morone saxatilis* were higher at lower water temperatures and recovery rate in *M. saxatilis* was more rapid at higher temperatures (Parma de Croux 1990, Gilderhus *et al.* 1991). A faster anaesthesia rate with an increase in water temperature was also observed in scallops, *Pecten fumatus* anaesthetized with chloral hydrate (Heasman *et al.* 1995).

The effect of temperature on the efficacy of anaesthetics is of primary importance in commercial abalone culture. The temperature at which abalone are cultured can be controlled to a certain extent with the aid of heating-cooling units. However, considering the quantities of water involved, maintaining water temperature at a constant level throughout the year would not be economical. Water temperature in South African abalone hatcheries and pilot grow-out facilities range from 9°C to 22°C (C. Claydon, Sea Plant Products, Hermanus, pers. comm. and C. Muller, Marine Growers, Port Elizabeth, pers. comm.).

Given the substantial range of temperatures at which abalone are cultured in South Africa, it was decided to investigate the effect of a range of temperatures (14, 16, 18 and 20°C) on the efficacy of magnesium sulphate ($MgSO_4$) and carbon dioxide (CO_2) as anaesthetics for three size classes of *Haliotis midae*. 2-Phenoxyethanol was not considered in this investigation because it caused mortalities during a growth trial (Chapter 5).

MATERIALS AND METHODS

Three size classes (5-15, 20-50 and 60-90 mm shell length (SL)) of *H. midae* were selected for this investigation. The small animals (5-15 mm SL) were obtained from the Sea Plant

Products hatchery in Hermanus, while larger animals (20-50 and 60-90 mm SL) were collected along the Eastern Cape Coast. Prior to the initiation of the experiments, the animals were acclimatized to laboratory conditions for a period of one month after which animals from each size class were acclimated to 14, 16, 18 and 20°C for two weeks. All experiments were conducted at a salinity of 35 ppt.

The effect of a range of concentrations of $MgSO_4$ (Table 4.1) and flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture (Table 4.2) were evaluated. Initial concentrations were based on the results described in Chapter 3. Each trial consisted of three replicates of 10 animals.

The trials were undertaken in plastic buckets containing 5 litres of continuously aerated seawater at the predetermined temperature. Ten animals were placed in each bucket and allowed to attach to the sides. To obtain the required dose ranges, $MgSO_4$ was weighed and dissolved in seawater. Once all the animals were firmly attached to the substratum, the seawater was poured out and replaced with the anaesthetic solution at exactly the same temperature. Anaesthesia with CO_2 was induced by bubbling the gas via a diffuser, through the seawater. The flow rate through the experimental chamber was controlled with a Messer Griesheim gas-flow regulator. O_2 and pH levels in the seawater were measured before commencing each run and once anaesthesia had been complete. The time taken for each animal to be completely anaesthetized was noted to the nearest 1 ± 0.5 sec. Complete anaesthesia was defined as the inability of the abalone to adhere onto the side or bottom of the experimental chamber. To terminate anaesthesia, the animals were transferred to a bucket containing fresh, aerated seawater at the same temperature and placed upside down on the bottom of the container. The time taken for each animal to revive was noted. Recovery was defined to be complete when the abalone turned right side up.

Statistical analysis

To analyze the effect of $MgSO_4$ concentration and CO_2 flow rate on anaesthesia and recovery rates, data were subjected to One-Way Analysis of Variance, with the main effects being concentration and flow rate. Means were compared using Tukey's Multiple Range Test at 5% error probability. Homogeneity of variances were tested with Bartlett's Test. When necessary, the natural logarithm transformation was used to stabilise the variances. Simple regression procedure was used to obtain the best model to predict anaesthesia rate as a function of concentration or flow rate at the various temperatures. The model with the

highest T-value for the slope, the highest F-ratio and most random residual pattern was chosen to be the best fit. When the model was found to be unsuitable for predicting anaesthesia rate as a function of concentration or flow rate, the data were subjected to the Kruskal-Wallis One-Way Analysis by Ranks procedure.

To test the effect of temperature on anaesthesia and recovery rate, data were subjected to Multifactor Analysis of Variance, with the main effects being temperature and concentration or flow rate. Means were compared using Tukey's Multiple Range test at 5% error probability. Homogeneity of variances were tested with Bartlett's Test. When necessary, the natural logarithm transformation was used to stabilise the variances.

The pH and O_2 levels measured during the CO_2 trials, were subjected to One-Way Analysis of Variance, with the main effects being flow rate. Means were compared using Tukey's Multiple Range Test. Homogeneity of variances were tested with Bartlett's Test (Zar 1984).

RESULTS

Exposure to different concentrations of $MgSO_4$ and continuous bubbling of the CO_2 gas mixture at different flow rates resulted in complete anaesthesia in all three size classes at all temperatures. The mean time to effect complete anaesthesia and recovery at each of the $MgSO_4$ concentrations and CO_2 flow rates at the various temperatures are shown in Tables 4.1 and 4.2. One-Way Analysis of Variance showed that there was an inverse relationship between $MgSO_4$ concentration and CO_2 flow rate and rate of anaesthesia at all four temperatures, i.e. the higher the concentration or flow rate, the more rapid the rate of anaesthesia. Statistical differences or similarities between mean anaesthesia rates for different concentrations and flow rates within the particular temperatures are shown in Tables 4.1 and 4.2 respectively, using different superscripts ($p<0.05$). The size of abalone was also positively correlated with effective $MgSO_4$ concentration and CO_2 flow rate. In each case higher concentrations were required for larger animals.

All the animals anaesthetized with $MgSO_4$ and CO_2 at the four temperatures recovered once they were placed in clean seawater. Statistical differences in recovery rates at different concentrations and flow rates are shown in Tables 4.1 and 4.2 respectively, using different superscripts ($p<0.05$). With the exception of a slight tendency towards a decrease in recovery rate with an increase in $MgSO_4$ concentration and CO_2 flow rate, there were no

apparent trends in the recovery rates within size classes. However, higher $MgSO_4$ concentrations and CO_2 flow rates were required for larger animals which resulted in substantially longer recovery times for the larger animals in comparison to recovery times for smaller animals. This tendency was especially evident when $MgSO_4$ was used.

Tables 4.3 and 4.4 show the effect of temperature on mean anaesthesia and recovery rates for all three size classes of *H. midae*. The statistical superscripts indicate differences or similarities between the various temperatures ($p < 0.05$). In general, there was an inverse relationship between temperature and rate of anaesthesia, i.e. the higher the temperature, the more rapid the rate of anaesthesia. There was no statistical difference in the rate of anaesthesia at 18°C and 20°C for 5-15 and 20-50 mm SL *H. midae* anaesthetized with CO_2.

Temperature also had an effect on recovery rate. The general tendency was for recovery rates to be more rapid at higher temperatures. There was no statistical difference in the rate of recovery from CO_2 anaesthesia at 18°C and 20°C for the two larger size classes. Similarly there was no statistical difference in the recovery rate from 12 g.100 ml^{-1} $MgSO_4$ anaesthesia of the 20-50 mm SL animals between 16°C and 18°C.

pH and O_2 levels recorded during the CO_2 anaesthesia trials are shown in Tables 4.5 and 4.6. Except for the tanks with the two smaller size classes animals subjected to a CO_2 flow rate of 6 $l.min^{-1}$ at 18°C and 20°C, and at 20°C, respectively, there were no significant differences in the O_2 concentration levels and pH. The changes in pH level in the seawater at 14°C and 16°C were slightly higher than the changes in pH level at 18°C and 20°C.

The predicted anaesthesia rate as a function of $MgSO_4$ concentration and CO_2 flow rate are shown in Figures 4.1 to 4.23. Anaesthesia rate could not be predicted as a function of $MgSO_4$ concentration for the 20-50 mm SL animals at 16°C. However, Kruskal-Wallis analysis showed that animals were anaesthetized significantly more rapid with 18 g.100 ml^{-1} $MgSO_4$
than with 12 g.100 ml^{-1} $MgSO_4$ ($p < 0.05$). These statistical differences were confirmed by One-Way Analysis of Variance (Table 4.1).

Table 4.1. The mean anaesthesia and recovery rates for three size classes of *Haliotis midae* after exposure to $MgSO_4$ at four temperatures. Standard deviations of the means also are presented. Different superscripts indicate significant differences in mean anaesthesia and recovery rates at $p < 0.05$ (note: although the same superscripts were used to denote statistical differences or similarities, anaesthesia and recovery rates were analyzed separately and should be interpreted as such).

Temp. °C	Size (mm SL)	Conc. g.100 ml^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)	Size (mm SL)	Conc. g.100 ml^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)	Size (mm SL)	Conc. g.100 ml^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)
14	5-15	2	33.9±14.7^{a}	15.9±6.4^{a}	20-50	12	15.6±7.0^{a}	39.2±8.8^{ab}	60-90	20	23.2±11.6^{a}	80.0±29.8^{a}
		4	6.6±4.7^{b}	9.9±4.3^{b}		14	11.3±5.5^{ab}	39.8±15.2^{a}		22	16.6±8.0^{ab}	62.8±24.9^{a}
		6	4.0±2.8^{b}	11.1±3.6^{ab}		16	7.8±4.5^{bc}	32.2±6.2^{b}		24	12.7±7.0^{bc}	^{b}
		8	1.9±1.4^{c}	11.8±6.9^{b}		18	6.0±3.3^{c}	18.9±9.2^{c}		26	8.9±4.5^{c}	53.6±26.6^{b}
												48.4±23.3^{b}
16	5-15	2	30.7±14.8^{d}	12.0±7.3^{c}	20-50	12	8.3±6.3^{d}	20.4±6.6^{d}	60-90	20	17.4±7.9^{d}	35.6±17.6^{c}
		4	3.4±3.4^{e}	10.8±5.2^{c}		14	6.6±4.7^{de}	20.9±10.1^{d}		22	13.7±6.1^{d}	^{d}
		6	2.0±1.7^{ef}	8.6±4.6^{cd}		16	6.2±4.6^{de}	22.9±11.1^{d}		24	8.3±3.3^{e}	49.3±22.9^{c}
		8	1.2±1.1^{f}	6.3±3.8^{d}		18	4.1±3.3^{e}	20.7±12.8^{d}		26	6.9±2.4^{e}	26.2±18.7^{d}
												^{e}
												15.6±10.6^{e}
18	5-15	2	25.0±15.8^{g}	8.5±3.9^{f}	20-50	6	18.5±8.8^{f}	26.5±4.0^{e}	60-90	14	47.8±19.3^{f}	134.8±18.6
		3	4.9±4.9^{h}	14.5±5.7^{e}		8	10.9±7.2^{g}	22.9±7.9^{ef}		16	38.7±9.6^{f}	^{f}
		4	2.0±2.2^{hi}	9.2±4.9^{f}		10	8.0±5.3^{g}	21.8±8.2^{ef}		18	30.3±15.2^{g}	77.2±27.4^{h}
		6	0.8±1.2^{i}	6.1±3.6^{g}		12	6.6±4.5^{gh}	18.0±8.5^{f}		20	25.0±13.2^{g}	85.3±14.6^{h}
		8	2.0±2.1^{hi}	8.9±4.1^{f}		14	5.2±5.0^{hi}	24.0±10.2^{e}		22	9.3±4.9^{h}	112.5±28.5
		10	0.3±0.3^{j}	2.9±2.0^{h}		16	3.8±2.3^{hi}	11.8±4.7^{g}		24	3.8±2.7^{i}	^{g}
						18	2.4±2.0^{i}	8.0±3.3^{h}				35.2±20.2^{i}
												22.1±13.0^{i}
20	5-15	1	8.6±7.1^{k}	2.2±2.5^{i}	20-50	8	3.0±2.0^{j}	3.7±3.0^{i}	60-90	18	9.3±5.0^{j}	50.0±20.6^{j}
		2	3.4±2.7^{l}	2.0±1.5^{ij}		10	1.7±0.9^{k}	3.6±1.6^{i}		20	7.8±3.3^{jk}	28.1±17.6^{k}
		3	2.6±1.8^{l}	0.9±1.1^{j}		12	1.0±0.5^{kl}	2.0±2.4^{j}		22	6.2±3.0^{k}	23.2±15.8^{k}
		4	1.4±1.0^{l}	1.1±0.8^{ij}		14	0.8±0.6^{l}	4.3±2.0^{i}		24	3.7±2.0^{l}	18.8±11.7^{k}

All animals recovered from anaesthesia once they were placed in fresh seawater and no post-recovery mortalities were recorded.

Table 4.2. The mean anaesthesia and recovery rates for three size classes of *Haliotis midae* after exposure to a CO_2 : O_2 gas mixture (11.3% : 88.7%) at four temperatures. Standard deviations of the means are also presented. Different superscripts indicate significant differences in mean anaesthesia and recovery rates at $p < 0.05$ (note: although the same superscripts were used to denote statistical differences or similarities, anaesthesia and recovery rates were analyzed separately and should be interpreted as such).

Temp. °C	Size (mm SL)	Flow rate l.min^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)	Size (mm SL)	Flow rate l.min^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)	Size (mm SL)	Flow rate l.min^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)
14	5-15	4	11.6 ± 8.6^{a}	8.6 ± 3.4^{a}	20-50	5	21.7 ± 14.7^{a}	11.3 ± 5.3^{a}	60-90	10	31.1 ± 15.4^{a}	16.9 ± 5.0^{a}
		5	6.7 ± 4.9^{b}	4.5 ± 4.0^{b}		6	12.1 ± 5.7^{b}	5.8 ± 3.2^{b}		12	22.3 ± 11.4^{ab}	14.8 ± 6.1^{a}
		6	5.8 ± 4.3^{bc}	2.7 ± 2.7^{b}		7	7.5 ± 5.3^{bc}	3.8 ± 2.2^{bc}		14	17.1 ± 8.3^{b}	10.0 ± 8.2^{b}
		7	3.7 ± 3.1^{c}	3.3 ± 3.0^{b}		8	3.9 ± 3.6^{c}	3.3 ± 2.6^{c}		16	10.0 ± 5.3^{c}	6.5 ± 4.9^{b}
16	5-15	4	6.5 ± 5.0^{d}	5.5 ± 2.9^{c}	20-50	5	10.8 ± 5.6^{d}	7.4 ± 3.3^{d}	60-90	10	17.1 ± 8.8^{d}	14.6 ± 4.6^{c}
		5	3.6 ± 2.4^{e}	2.4 ± 2.0^{d}		6	6.6 ± 3.7^{dc}	3.3 ± 3.1^{e}		12	11.8 ± 5.8^{dc}	4.4 ± 3.0^{d}
		6	2.7 ± 2.2^{ef}	1.4 ± 1.2^{d}		7	2.8 ± 2.0^{ef}	2.4 ± 1.6^{ef}		14	8.4 ± 5.5^{ef}	3.2 ± 2.0^{d}
		7	2.2 ± 1.9^{f}	1.5 ± 1.1^{d}		8	1.8 ± 2.0^{f}	1.3 ± 1.1^{f}		16	6.7 ± 5.8^{f}	3.4 ± 2.7^{d}
18	5-15	3	5.9 ± 4.4^{g}	3.0 ± 2.8^{e}	20-50	2	10.2 ± 8.7^{g}	3.8 ± 1.9^{g}	60-90	6	12.6 ± 9.6^{g}	5.7 ± 3.5^{e}
		4	3.1 ± 2.3^{g}	2.7 ± 2.6^{e}		3	5.6 ± 4.1^{gh}	4.0 ± 2.0^{g}		8	8.5 ± 3.6^{g}	2.8 ± 2.7^{f}
		5	1.6 ± 1.3^{h}	1.8 ± 1.7^{e}		4	2.7 ± 1.7^{h}	0.6 ± 0.3^{h}		10	4.5 ± 2.8^{h}	2.6 ± 1.6^{f}
		6	0.6 ± 0.6^{i}	1.1 ± 0.6^{e}		5	1.2 ± 1.0^{i}	0.8 ± 0.5^{h}		12	2.6 ± 2.1^{h}	1.9 ± 1.3^{f}
						6	1.1 ± 0.9^{i}	0.9 ± 0.5^{h}				
						7	0.8 ± 0.7^{i}	0.7 ± 0.4^{h}				
20	5-15	3	4.0 ± 2.8^{j}	1.3 ± 1.3^{fg}	20-50	3	5.2 ± 4.0^{j}	2.1 ± 1.5^{i}	60-90	6	8.8 ± 7.7^{i}	5.4 ± 3.3^{g}
		4	3.2 ± 2.4^{j}	2.4 ± 3.0^{f}		4	2.2 ± 1.5^{k}	0.9 ± 0.7^{j}		8	6.1 ± 5.3^{i}	4.3 ± 3.3^{gh}
		5	1.3 ± 1.1^{k}	1.0 ± 1.4^{gh}		5	1.2 ± 0.7^{kl}	0.6 ± 0.6^{j}		10	4.1 ± 2.8^{i}	2.5 ± 2.1^{hi}
		6	0.5 ± 0.4^{k}	0.6 ± 0.6^{h}		6	0.9 ± 0.6^{l}	0.6 ± 0.6^{j}		12	1.8 ± 1.4^{j}	1.6 ± 0.9^{i}

All animals recovered from anaesthesia once they were placed in fresh seawater and no post-recovery mortalities were recorded.

Table 4.3. The effect of temperature on the mean anaesthesia and recovery rates for three size classes of *Haliotis midae* after exposure to $MgSO_4$. Standard deviations of the means are also presented. Different superscripts indicate significant differences in mean anaesthesia and recovery rates at different temperatures at $p < 0.05$ (note: although the same superscripts were used to denote statistical differences or similarities, anaesthesia and recovery rates were analyzed separately and should be interpreted as such).

Temp °C	Size (mm SL)	Conc. g.100 ml^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)	Size (mm SL)	Conc. g.100 ml^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)	Size (mm SL)	Conc. g.100 ml^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)
14	5-15	2	33.9 ± 14.7^{a}	15.9 ± 6.4^{a}	20-50	12	15.6 ± 7.0^{a}	39.2 ± 8.8^{ab}	60-90	20	23.2 ± 11.6^{a}	80.0 ± 29.8^{a}
		4	6.6 ± 4.7^{b}	9.9 ± 4.3^{b}		14	11.3 ± 5.5^{ab}	39.8 ± 15.2^{a}		22	16.6 ± 8.0^{ab}	62.8 ± 24.9^{ab}
		6	4.0 ± 2.8^{b}	11.1 ± 3.6^{ab}		16	7.8 ± 4.5^{bc}	32.2 ± 6.2^{b}		24	12.7 ± 7.0^{bc}	53.6 ± 26.6^{b}
		8	1.9 ± 1.4^{c}	11.8 ± 6.9^{b}		18	6.0 ± 3.3^{c}	18.9 ± 9.2^{c}		26	8.9 ± 4.5^{c}	48.4 ± 23.3^{b}
16	5-15	2	30.7 ± 14.8^{d}	12.0 ± 7.3^{c}	20-50	12	8.3 ± 6.3^{d}	20.4 ± 6.6^{d}	60-90	20	17.4 ± 7.9^{d}	35.6 ± 17.6^{cd}
		4	3.4 ± 3.4^{e}	10.8 ± 5.2^{c}		14	6.6 ± 4.7^{de}	20.9 ± 10.1^{d}		22	13.7 ± 6.1^{d}	49.3 ± 22.9^{c}
		6	2.0 ± 1.7^{ef}	8.6 ± 4.6^{cd}		16	6.2 ± 4.6^{de}	22.9 ± 11.1^{d}		24	8.3 ± 3.3^{e}	26.2 ± 18.7^{d}
		8	1.2 ± 1.1^{f}	6.3 ± 3.8^{d}		18	4.1 ± 3.3^{e}	20.7 ± 12.8^{d}		26	6.9 ± 2.4^{e}	15.6 ± 10.6^{e}
18	5-15	2	25.0 ± 15.8^{g}	8.5 ± 3.9^{f}	20-50	8	10.9 ± 7.2^{f}	22.9 ± 7.9^{de}	60-90	18	30.3 ± 15.2^{g}	85.3 ± 14.6^{g}
		3	4.9 ± 4.9^{h}	14.5 ± 5.7^{e}		10	8.0 ± 5.3^{f}	21.8 ± 8.2^{de}		20	25.0 ± 13.2^{g}	112.5 ± 28.5^{f}
		4	2.0 ± 2.2^{hi}	9.2 ± 4.9^{f}		12	6.6 ± 4.5^{fg}	18.0 ± 8.5^{d}		22	9.3 ± 4.9^{h}	35.2 ± 20.2^{h}
		6	0.8 ± 1.2^{i}	6.1 ± 3.6^{g}		14	5.2 ± 5.0^{gh}	24.0 ± 10.2^{e}		24	3.8 ± 2.7^{i}	22.1 ± 13.0^{h}
		8	2.0 ± 2.1^{hi}	8.9 ± 4.1^{df}		16	3.8 ± 2.3^{gh}	11.8 ± 4.7^{f}				
						18	2.4 ± 2.0^{h}	8.0 ± 3.3^{g}				
20	5-15	2	3.4 ± 2.7^{j}	2.0 ± 1.5^{h}	20-50	8	3.0 ± 2.0^{i}	3.7 ± 3.0^{h}	60-90	18	9.3 ± 5.0^{j}	50.0 ± 20.6^{i}
		3	2.6 ± 1.8^{ij}	0.9 ± 1.1^{i}		10	1.7 ± 0.9^{ij}	3.6 ± 1.6^{h}		20	7.8 ± 3.3^{jk}	28.1 ± 17.6^{ij}
		4	1.4 ± 1.0^{ij}	1.1 ± 0.8^{hi}		12	1.0 ± 0.5^{ik}	2.0 ± 2.4^{i}		22	6.2 ± 3.0^{k}	23.2 ± 15.8^{ij}
						14	0.8 ± 0.6^{k}	4.3 ± 2.0^{h}		24	3.7 ± 2.0^{i}	18.8 ± 11.7^{ij}

Table 4.4. The effect of temperature on the mean anaesthesia and recovery rates for three size classes of *Haliotis midae* after exposure to a CO_2 : O_2 gas mixture (11.3% : 88.7%). Standard deviations of the means are also presented. Different superscripts indicate significant differences in mean anaesthesia and recovery rates at different temperatures at $p < 0.05$ (note: although the same superscripts were used to denote statistical differences or similarities, anaesthesia and recovery rates were analyzed separately and should be interpreted as such).

Temp. °C	Size (mm SL)	Flow rate l.min^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)	Size (mm SL)	Flow rate l.min^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)	Size (mm SL)	Flow rate l.min^{-1}	Mean rate of anaesthesia (min)	Mean recovery rate (min)
14	5-15	4	11.6±8.6^{a}	8.6±3.4^{a}	20-50	5	21.7±14.7^{a}	11.3±5.3^{a}	60-90	10	31.1±15.4^{a}	16.9±5.0^{a}
		5	6.7±4.9^{b}	4.5±4.0^{b}		6	12.1±5.7^{b}	5.8±3.2^{b}		12	22.3±11.4^{ab}	14.8±6.1^{a}
		6	5.8±4.3^{bc}	2.7±2.7^{b}		7	7.5±5.3^{bc}	3.8±2.2^{bc}		14	17.1±8.3^{b}	10.0±8.2^{b}
		7	3.7±3.1^{c}	3.3±3.0^{b}		8	3.9±3.6^{c}	3.3±2.6^{c}		16	10.0±5.3^{c}	6.5±4.9^{b}
16	5-15	4	6.5±5.0^{d}	5.5±2.9^{c}	20-50	5	10.8±5.6^{d}	7.4±3.3^{d}	60-90	10	17.1±8.8^{d}	14.6±4.6^{c}
		5	3.6±2.4^{e}	2.4±2.0^{d}		6	6.6±3.7^{dc}	3.3±3.1^{e}		12	11.8±5.8^{de}	4.4±3.0^{d}
		6	2.7±2.2^{ef}	1.4±1.2^{d}		7	2.8±2.0^{ef}	2.4±1.6^{ef}		14	8.4±5.5^{ef}	3.2±2.0^{d}
		7	2.2±1.9^{f}	1.5±1.1^{d}		8	1.8±2.0^{f}	1.3±1.1^{f}		16	6.7±5.8^{f}	3.4±2.7^{d}
18	5-15	3	5.9±4.4^{g}	3.0±2.8^{e}	20-50	3	5.6±4.1^{gh}	4.0±2.0^{g}	60-90	6	12.6±9.6^{g}	5.7±3.5^{e}
		4	3.1±2.3^{g}	2.7±2.6^{e}		4	2.7±1.7^{h}	0.6±0.3^{h}		8	8.5±3.6^{g}	2.8±2.7^{f}
		5	1.6±1.3^{h}	1.8±1.7^{e}		5	1.2±1.0^{i}	0.8±0.5^{h}		10	4.5±2.8^{h}	2.6±1.6^{f}
		6	0.6±0.6^{h}	1.1±0.6^{e}		6	1.1±0.9^{i}	0.9±0.5^{h}		12	2.6±2.1^{h}	1.9±1.3^{f}
						7	0.8±0.7^{i}	0.7±0.4^{h}				
20	5-15	3	4.0±2.8^{g}	1.3±1.3^{fg}	20-50	3	5.2±4.0^{gh}	2.1±1.5^{i}	60-90	6	8.8±7.7^{i}	5.4±3.3^{e}
		4	3.2±2.4^{g}	2.4±3.0^{g}		4	2.2±1.5^{h}	0.9±0.7^{h}		8	6.1±5.3^{i}	4.3±3.3^{ef}
		5	1.3±1.1^{h}	1.0±1.4^{gh}		5	1.2±0.7^{i}	0.6±0.6^{h}		10	4.1±2.8^{i}	2.5±2.1^{f}
		6	0.5±0.4^{h}	0.6±0.6^{h}		6	0.9±0.6^{i}	0.6±0.6^{h}		12	1.8±1.4^{j}	1.6±0.9^{f}

Table 4.5. The mean pH and O_2 levels measured in the test tanks before and after anaesthesia of three size classes of *Haliotis midae* at 14°C and 16°C with a CO_2 : O_2 gas mixture (11.3% : 88.7%). Different superscripts indicate significant differences in mean pH and O_2 level changes at $p<0.05$.

Temp. °C	Size (mm SL)	Flow rate l.min^{-1}	Mean total exposure time to the gas mixture (min)	Mean O_2 concentration mg.l^{-1}			Mean pH		
				Start of run	End of run	Change	Start of run	End of run	Change
14	5-15	4	28.64	9.10	34.50^{a}	25.40^{f}	7.86	5.75^{l}	2.11^{st}
		5	15.50	8.87	34.43^{a}	25.57^{f}	7.82	5.75^{l}	2.06^{st}
		6	12.47	9.03	34.53^{a}	25.50^{f}	7.85	5.76^{l}	2.09^{st}
		7	9.08	8.97	34.50^{a}	25.53^{f}	7.84	5.77^{lm}	2.07^{st}
14	20-50	5	47.86	9.20	34.77^{a}	25.57^{f}	7.91	5.81^{lmn}	2.10^{st}
		6	21.34	9.13	34.70^{a}	25.57^{f}	7.89	5.80^{lmn}	2.09^{st}
		7	16.74	9.13	34.57^{a}	25.43^{f}	7.81	5.76^{l}	2.05^{s}
		8	11.94	9.00	34.50^{a}	25.50^{f}	7.82	5.78^{lm}	2.05^{s}
14	60-90	10	60.11	8.47	33.87^{a}	25.40^{f}	7.95	5.79^{lm}	2.16^{st}
		12	44.00	8.57	33.60^{a}	25.03^{f}	7.92	5.80^{lmn}	2.12^{st}
		14	30.37	8.63	33.73^{a}	25.10^{f}	7.93	5.82^{lmn}	2.11^{st}
		16	18.64	8.47	33.70^{a}	25.23^{f}	7.89	5.77^{l}	2.14^{st}
16	5-15	4	17.36	9.17	34.53^{a}	25.37^{f}	7.86	5.79^{lm}	2.08^{st}
		5	7.84	9.13	34.60^{a}	25.47^{f}	7.87	5.80^{lmn}	2.07^{st}
		6	7.44	9.23	34.57^{a}	25.33^{f}	7.89	5.82^{lmn}	2.07^{st}
		7	6.40	9.03	34.10^{a}	25.07^{f}	7.87	5.81^{lmn}	2.06^{st}
16	20-50	5	21.84	9.03	34.33^{a}	25.30^{f}	7.87	5.78^{lm}	2.09^{st}
		6	14.54	9.00	34.63^{a}	25.63^{f}	7.89	5.79^{lm}	2.10^{st}
		7	6.65	9.00	34.27^{a}	25.27^{f}	7.87	5.83^{lmn}	2.04^{s}
		8	5.87	8.80	34.20^{a}	25.40^{f}	7.88	5.85^{lmn}	2.04^{s}
16	60-90	10	33.59	8.53	33.73^{a}	25.20^{f}	7.95	5.86^{lmn}	2.09^{st}
		12	21.02	8.90	34.27^{a}	25.37^{f}	7.92	5.80^{lmn}	2.12^{st}
		14	16.45	8.53	33.80^{a}	25.27^{f}	7.96	5.87^{lmn}	2.10^{st}
		16	16.12	8.57	33.73^{a}	25.17^{f}	7.95	5.84^{lmn}	2.11^{st}

DISCUSSION

Both $MgSO_4$ and the CO_2 gas mixture proved to be effective anaesthetics for *H. midae* over the entire range of temperatures. Both the chemical and the gas mixture met the criteria of an effective abalone anaesthetic at all temperatures as they induced rapid and mortality-free anaesthesia in all three size classes. It is suggested that the inverse relationship between $MgSO_4$ concentration and CO_2 flow rate and the rate of anaesthesia at all temperatures was probably due to a more rapid absorption rate of the anaesthetic at the higher concentrations

Table 4.6. The mean pH and O_2 levels measured in the test tanks before and after anaesthesia of three size classes of *Haliotis midae* at 18°C and 20°C with a CO_2 : O_2 gas mixture (11.3% : 88.7%). Different superscripts indicate significant differences in mean pH and O_2 level changes at $p<0.05$.

Temp °C	Size (mm SL)	Flow rate l.min^{-1}	Mean total exposure time to the gas mixture (min)	Mean O_2 concentration mg.l^{-1}			Mean pH		
				Start of run	End of run	Change	Start of run	End of run	Change
18	5-15	3	13.62	8.67	34.00^{a}	25.33^{f}	7.91	5.91^{mn}	2.01^{s}
		4	7.06	8.67	33.97^{a}	25.30^{f}	7.88	5.93^{n}	1.95^{s}
		5	3.61	8.63	33.77^{a}	25.13^{f}	7.89	5.90^{mn}	1.99^{s}
		6	1.69	8.47	25.87^{e}	17.40^{j}	7.92	6.21^{o}	1.71^{q}
18	20-50	2	23.76	8.97	34.17^{a}	25.20^{f}	7.90	5.86^{lmn}	2.04^{s}
		3	13.15	8.73	34.00^{a}	25.27^{f}	7.89	5.90^{mn}	1.99^{s}
		4	5.31	8.83	34.07^{a}	25.23^{f}	7.86	5.86^{lmn}	2.00^{s}
		5	3.02	8.80	33.90^{a}	25.10^{f}	7.87	5.88^{lmn}	1.99^{s}
		6	2.44	8.67	33.73^{a}	25.07^{f}	7.89	5.90^{mn}	1.99^{s}
		7	1.98	8.77	33.03^{b}	24.27^{g}	7.88	5.92^{mn}	1.96^{s}
18	60-90	6	30.92	8.77	34.13^{a}	25.37^{f}	7.86	5.88^{lmn}	1.98^{s}
		8	14.08	8.50	34.13^{a}	25.63^{f}	7.92	5.92^{mn}	2.00^{s}
		10	8.79	8.87	34.40^{a}	25.53^{f}	7.85	5.83^{lmn}	2.02^{s}
		12	6.48	8.53	33.93^{a}	25.40^{f}	7.91	5.88^{lmn}	2.03^{s}
20	5-15	3	8.17	8.83	34.13^{a}	25.30^{f}	7.89	5.89^{lmn}	2.00^{s}
		4	6.99	8.87	34.10^{a}	25.23^{f}	7.87	5.89^{lmn}	1.98^{s}
		5	3.35	8.83	34.10^{a}	25.27^{f}	7.84	5.88^{lmn}	1.96^{s}
		6	1.29	9.07	27.73^{d}	18.67^{i}	7.87	6.22^{o}	1.64^{p}
20	20-50	3	12.02	9.07	34.33^{a}	25.27^{f}	7.88	5.91^{mn}	1.97^{s}
		4	4.73	9.13	34.17^{a}	25.03^{f}	7.89	5.89^{mn}	2.00^{s}
		5	2.38	8.83	33.77^{a}	24.93^{f}	7.89	5.89^{mn}	2.00^{s}
		6	1.98	9.17	31.33^{c}	22.17^{h}	7.88	5.98^{n}	1.90^{r}
20	60-90	6	22.48	8.70	34.13^{a}	25.43^{f}	7.82	5.82^{lmn}	2.00^{s}
		8	15.14	8.93	34.50^{a}	25.57^{f}	7.94	5.90^{mn}	2.04^{s}
		10	8.60	8.87	34.23^{a}	25.37^{f}	7.89	5.88^{lmn}	2.01^{s}
		12	4.07	8.67	33.77^{a}	25.10^{f}	7.91	5.88^{lmn}	2.03^{s}

and flow rates. As expected, there was an inverse relationship between temperature and rate of anaesthesia. The rate of anaesthesia was more rapid at higher temperatures. Therefore, higher $MgSO_4$ concentrations and CO_2 flow rates were required at the lower temperatures. It was hypothesized that the inverse relationship between rate of anaesthesia and temperature was due to an increase in metabolic and heart rate. In fish, metabolic rates have been shown to increase with an increase in temperature (Pickering 1992, Teo & Chen 1993) and heart

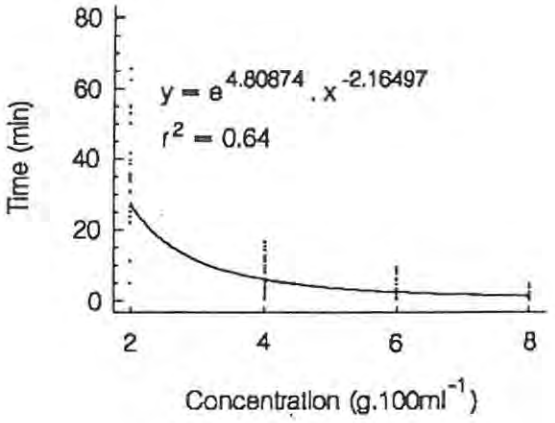


Figure 4.1. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 14°C.

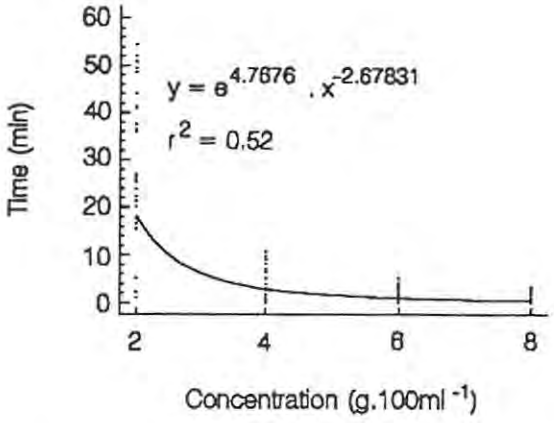


Figure 4.2. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 16°C.

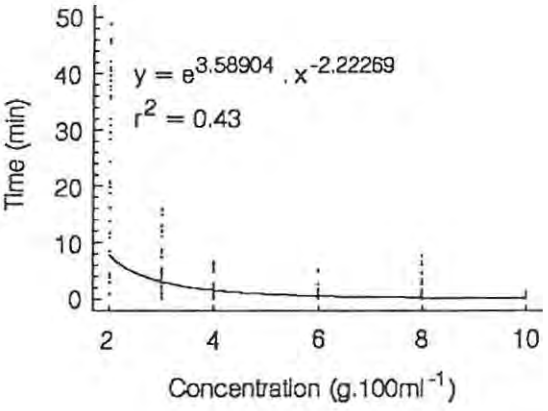


Figure 4.3. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 18°C.

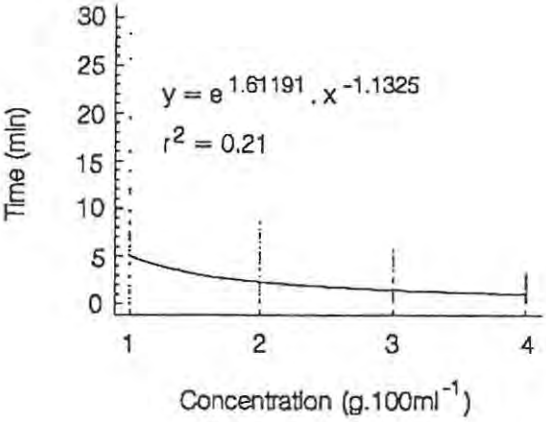


Figure 4.4. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 20°C.

rate of abalone has also been shown to increase with an increase in temperature up to a maximum of approximately 30°C after which it declines sharply to zero beats per minute (Fujino *et al.* 1984, Hahn 1989d). An increase in the metabolic rate and the heart rate at higher temperatures could possibly lead to an enhanced rate of uptake of the anaesthetic.

The faster recovery rates at higher temperatures was suggested to be a possible consequence of the higher metabolic and heart rates at the higher temperatures, which probably enhanced

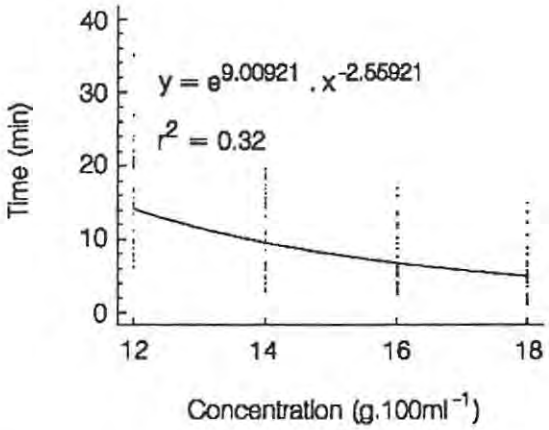


Figure 4.5. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 14°C.

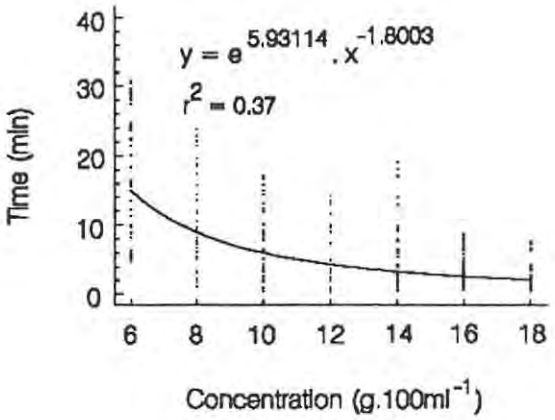


Figure 4.6. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 18°C.

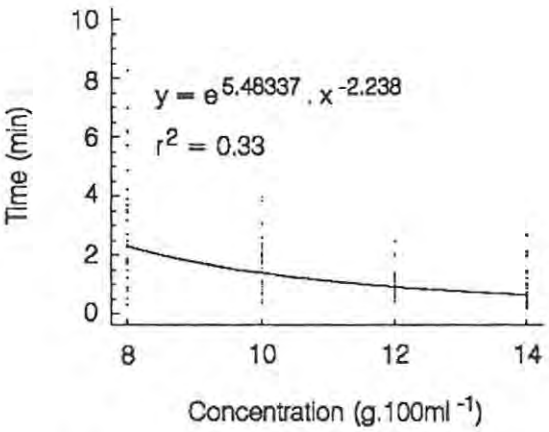


Figure 4.7. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 20°C.

the elimination rate of the anaesthetic from the body. Also, less $MgSO_4$ was required at higher temperatures which meant that less had to be eliminated from the body of the animal.

The slightly greater changes in pH noted with CO_2 anaesthesia at 14°C and 16°C in comparison to 18°C and 20°C is a direct result of the greater solubility of CO_2 in seawater at lower temperatures (Strickland & Parsons 1972, Colt 1984). The greater the partial pressure of CO_2 in the seawater, the greater the decrease in pH (Spotte 1979). The significantly smaller changes in O_2 and pH levels at a flow rate of 6 $l.min^{-1}$ at 18°C and

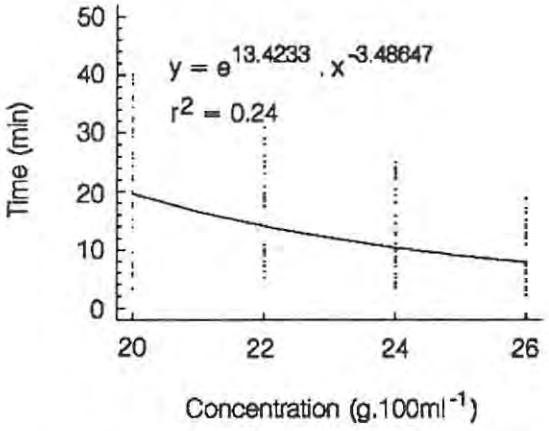


Figure 4.8. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 14°C.

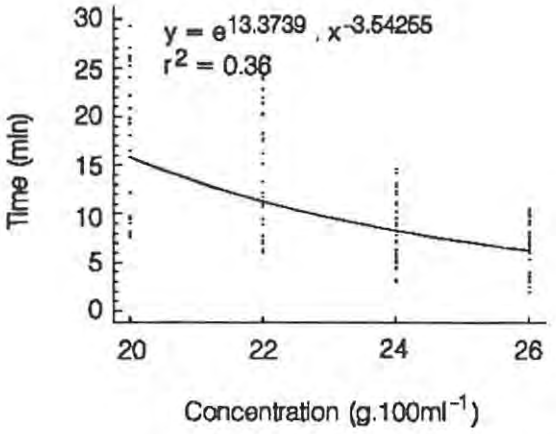


Figure 4.9. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 16°C.

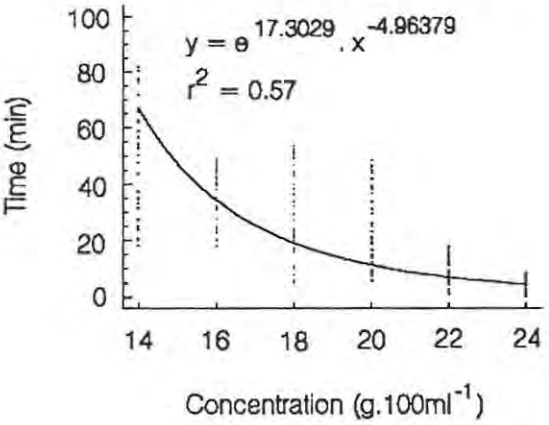


Figure 4.10. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 18°C.

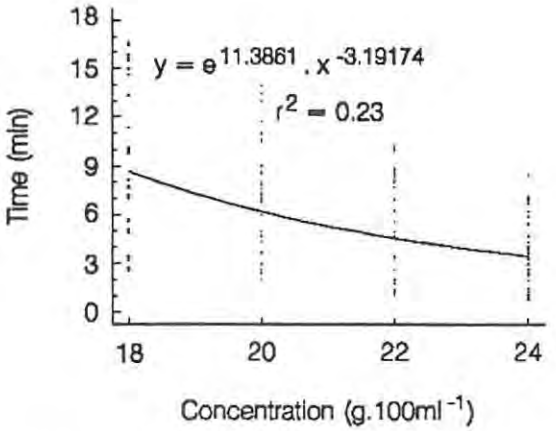


Figure 4.11. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different concentrations of $MgSO_4$ at 20°C.

20°C in the 5-15 mm SL size class and at 20°C in the 20-50 mm SL size class were probably due to the significantly shorter exposure time to the CO_2 gas mixture at these flow rates and temperatures. The expected trend would be for the rate of anaesthesia to be more rapid at lower temperatures, since the pH changes at lower temperatures were greater. However, despite the greater change in pH at lower temperatures, there was an inverse correlation between temperature and anaesthesia rate. The inverse relationship between temperature and rate of CO_2 anaesthesia was probably due to an increase in metabolic and heart rate at higher temperatures, as suggested above. Therefore, despite the higher partial

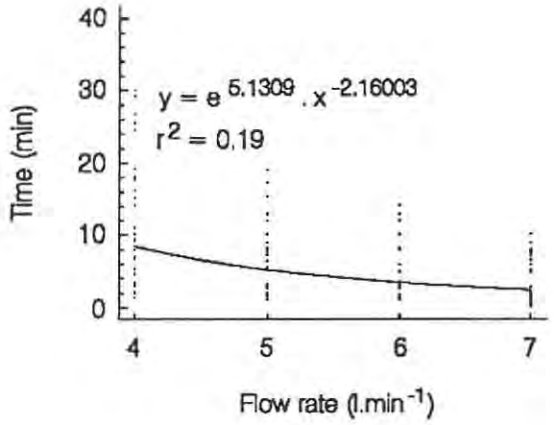


Figure 4.12. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 14°C.

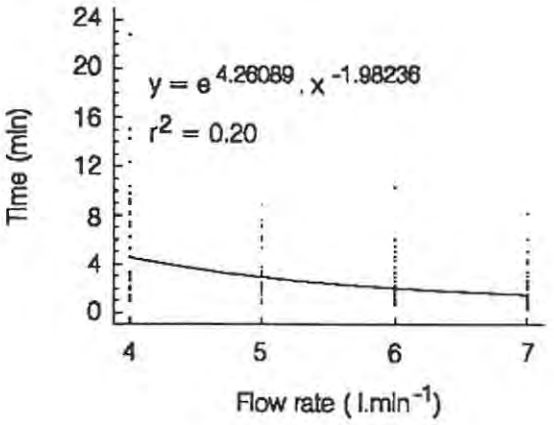


Figure 4.13. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 16°C.

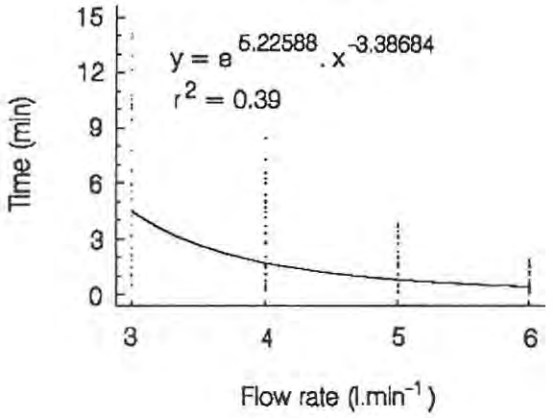


Figure 4.14. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 18°C.

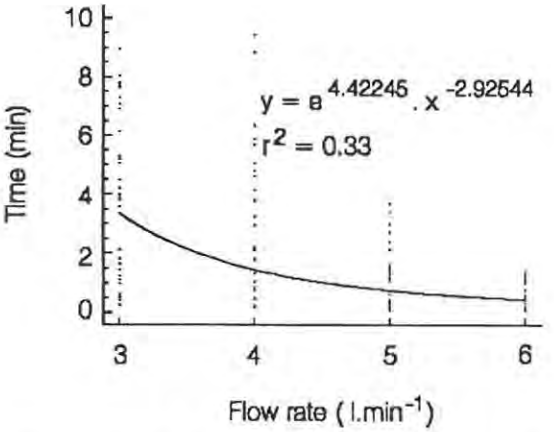


Figure 4.15. The predicted anaesthesia rates for 5-15 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 20°C.

pressures of CO_2 at lower temperatures, the theoretical increase in metabolic and heart rate at higher temperatures possibly increased the rate of absorption of CO_2. This in turn may have resulted in a more rapid decrease in intracellular pH, the effects of which are described in Chapter 3. The more rapid recovery rates from CO_2 anaesthesia at higher temperatures can possibly be ascribed to a more rapid normalization of enzyme (and therefore muscle) activity as a direct result of increased metabolic and heart rate. Given the longer exposure times to higher partial pressures of CO_2 at the lower temperatures would probably have

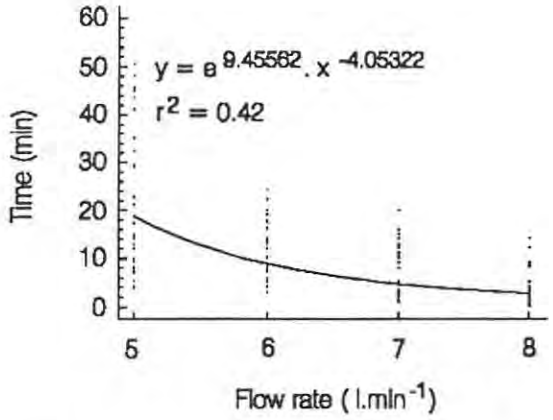


Figure 4.16. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 14°C.

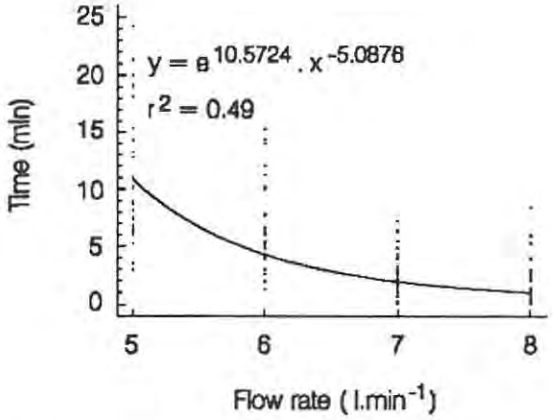


Figure 4.17. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 16°C.

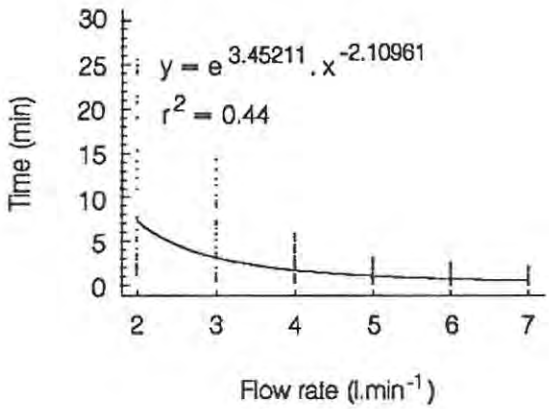


Figure 4.18. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 18°C.

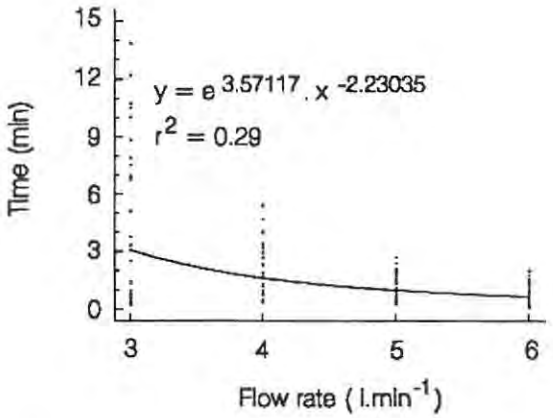


Figure 4.19. The predicted anaesthesia rates for 20-50 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 20°C.

lowered the internal pH level to a greater extent than in animals exposed to CO_2 at higher temperatures, resulting in the longer recovery rates. Sugiyama and Tanaka (1982) also noted a decrease in anaesthesia and recovery rates of abalone exposed to CO_2 anaesthesia at higher temperatures.

In conclusion, the data obtained in this study provides the commercial abalone farmer with information concerning the interrelationships between temperature and concentration of $MgSO_4$ and flow rate of a CO_2 gas mixture on the rate of anaesthesia. The dosage tables and

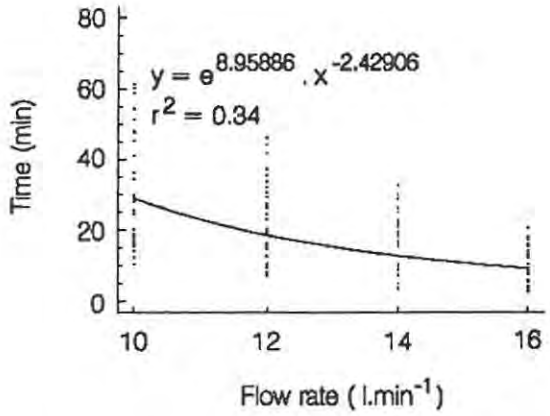


Figure 4.20. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 14°C.

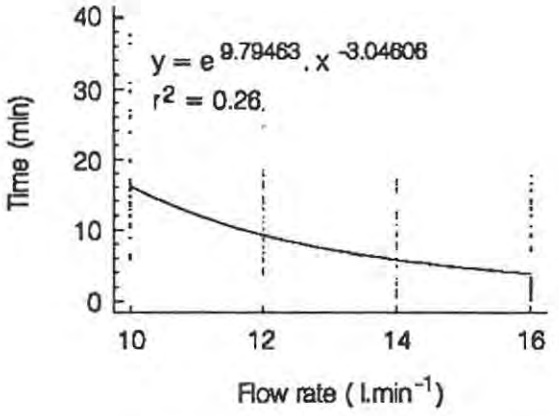


Figure 4.21. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 16°C.

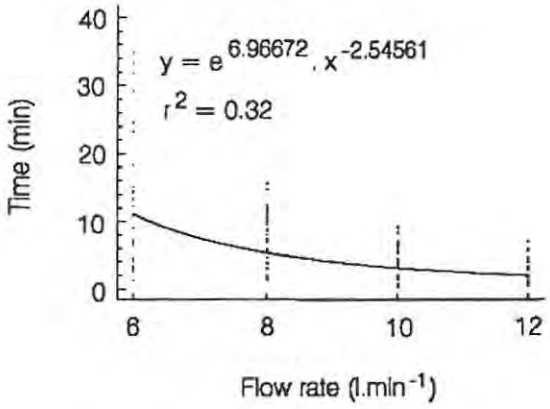


Figure 4.22. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 18°C.

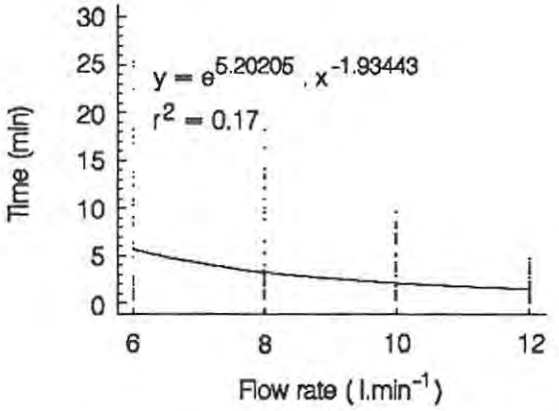


Figure 4.23. The predicted anaesthesia rates for 60-90 mm SL *Haliotis midae* at different flow rates of an 11.3% CO_2 : 88.7% O_2 gas mixture at 20°C.

regression models can be used as guidelines to predict anaesthesia rates for different size animals as a function of temperature.

The recommended dosage rates described in this, and in the previous chapter, were tested on a commercial scale at the Sea Plant Products abalone hatchery in Hermanus in April 1995. CO_2 anaesthesia was found to be impractical. However, 12 000 juvenile abalone (12-13 mm SL) were successfully anaesthetized with $MgSO_4$ using the size and temperature related dosage tables developed in this study (H.I. White & C. Claydon, unpublished data).

CHAPTER 5

EFFECTS OF LONG TERM INTERMITTENT MAGNESIUM SULPHATE AND 2-PHENOXYETHANOL ANAESTHESIA ON *HALIOTIS MIDAE* GROWTH AND MORTALITY

INTRODUCTION

Finding a chemical which results in mortality-free anaesthesia, does not completely fulfil the criteria of an effective anaesthetic. Another very important consideration is the long term sub-lethal effects of regular anaesthesia on growth.

Abalone have very slow growth rates. Depending on ambient temperatures it would theoretically take between 3 to 4.8 years to rear abalone to 80 mm shell length (SL) (90-100 g) on the South African coast (Hecht 1992). Rapid grow-out to commercial size is therefore an important financial consideration. Hampering growth even further would be detrimental to the commercial abalone farmer and it is essential that regular anaesthesia during routine farming procedures should not have an effect on growth rate.

No information on the effect of anaesthesia on growth of aquatic organisms, except for the effect of methanesulfonate (MS-222) on cupular growth rate of free neuromasts in the larvae of three species of cyprinid fish (Mukai & Kobayashi 1992), has been documented to date. Mukai and Kobayashi (1992) found that the exposure of the fish to MS-222 did not affect the growth of the cupulae.

In this study, the effect of regular anaesthesia on the growth of three size classes of *Haliotis midae* was evaluated. Two chemicals, magnesium sulphate ($MgSO_4$) and 2-phenoxyethanol, were selected for this growth experiment as both chemicals exhibited good anaesthetic potential in previous investigations (Chapter 2 and 3). The effect of carbon dioxide (CO_2) anaesthesia on growth was not included in this study, since it has been shown to be impractical and too costly for *H. midae* anaesthesia on a commercial scale (H.I. White and C. Claydon, unpublished data).

MATERIALS AND METHODS

Three size classes (5-15, 20-50 and 60-90 mm SL) of *H. midae* were selected for this study. The small animals (5-15 mm SL) were obtained from the Sea Plant Products hatchery in

Hermanus, while larger animals (20-50 and 60-90 mm SL) were collected along the Eastern Cape Coast. Prior to the initiation of the experiments, the animals were acclimated to laboratory conditions at the Port Alfred laboratory for a period of one month.

The animals were reared in 30.5 cm x 31 cm x 38 cm glass aquaria linked to a 5000 l recirculating system Figure (5.1). Twenty percent of the water in the system was replaced on a daily basis. Biologically filtered seawater was circulated through each aquarium at a flow rate of 0.6 l/min. The water in each aquarium was aerated continuously and temperature in the system was maintained at 18.6°C$\pm$1.1°C, with the aid of a heating-cooling unit, throughout the eight month growth trial. This temperature simulates the average annual temperature along the Eastern Cape Coast (Port Alfred Marine Laboratory records). Salinity varied between 33 and 35 ppt (mean 34.6 ppt$\pm$0.7 ppt). Photoperiod was controlled at $\pm$ 12 L: 12 D. The animals were fed with an extruded pellet supplied by Sea Plant Products in Hermanus (proximate pellet composition is given in Table 5.1). The food was fed at a ratio of 2% of body weight for the 5-15 mm SL animals and 4% of body weight for the 20-50 and 60-90 mm SL animals at 16h00 every second day.

There were three replicate groups per treatment per size class and three control replicate groups per size class. Thirty animals were used in each replicate for the 5-15 and 20-50 mm SL size classes and ten in each replicate for the 60-90 mm SL size class. All animals in the 20-50 and 60-90 mm SL size classes were tagged individually (5-15 mm SL animals were too small for tagging purposes). Except for the control group the animals were anaesthetized, weighed and measured monthly over a period of eight months. To induce anaesthesia, the water supply was turned off and all the water was drained from the aquarium. The concentrations of $MgSO_4$ and 2-phenoxyethanol used to induce anaesthesia in each size class were selected from Table 3.1. To obtain the required dosages, $MgSO_4$ was weighed and 2-phenoxyethanol volumetrically measured, and dissolved in 4 l of seawater. The anaesthetic solution was then poured into the appropriate aquarium and once all the animals had been anaesthetized, the time taken for complete anaesthesia of the all animals in each tank was noted. The animals were then removed from the tanks and weighed individually to the nearest 0.01 g and shell lengths were measured to the nearest 0.1 mm with a vernier calliper. After weighing and measuring, the anaesthetic solution in the aquarium was replaced with fresh,

Figure 5.1. Photograph of aquaria used to house *Haliotis midae* during the eight month growth trial. A = water supply, B = air supply, C = drainage pipes, D = holding tanks.

Table 5.1. Proximate composition of the artificial diet used for *Haliotis midae* during the eight month growth trial.

Ingredient	%
Protein	34.6
Carbohydrates	43.3
Fat	5.3
Crude fibre	1.2
Ash	5.7
Moisture	10.0

Energy content: 14,9 kJ/g.

filtered seawater, the water supply was turned on and the animals were placed upside down in the aquarium. The total time taken for all the animals in the aquarium to revive was noted. Recovery was defined to be complete when all the abalone had turned right side up. Animals from the control group were simply levered off, individually weighed and measured, and replaced in the aquarium. Mortalities were recorded on a daily basis throughout the eight month period.

Statistical analysis

The total anaesthesia rates, recovery rates and exposure time to air during monthly weighing and measuring, were subjected to One-Way Analysis of Variance, with the main effect being temperature. Means were compared using Tukey's Multiple Range Test at 5% error probability. Homogeneity of variances were tested with Bartlett's Test (Zar 1984).

The weights and shell lengths of each treatment and size class were subjected to the Simple Regression procedure to obtain the best model with which to predict growth rate as a function of time for each treatment and size class. The model with the highest T-value for the slope, the highest F-ratio and the most random residual pattern was chosen to be the best fit. To compare the growth rates between treatments, the data was linearized by using the natural logarithm transformations of both the x and y variable, since the best fit obtained from the Simple Regression procedure (the multiplicative model) was not linear. To test for significant differences in the slopes obtained from the Simple Regression procedure, the data were subjected to the Multiple Regression procedure after which the residual sum of squares obtained from the Simple Regression procedure and the error term obtained from the Multiple Regression procedure, were subjected to the F-test (Zar 1984). The data obtained from the 2-phenoxyethanol treated animals were not subjected to regression analysis, since percentage monthly mortalities were too high (Table 5.4, Figures 5.2 to 5.4).

RESULTS

There were no statistical differences in total anaesthesia rates, recovery rates and exposure time to air during monthly weighing and measuring within all size classes of *H. midae* treated with $MgSO_4$, except for anaesthesia rates in the largest size class (60-90 mm SL) (Table 5.2). Mean total anaesthesia rates of the 60-90 mm SL animals treated with 24 g.100 ml^{-1} $MgSO_4$ appeared to decrease with an increase in temperature.

Mean total anaesthesia rates in the 5-15 and the 20-50 mm SL animals treated with 2-phenoxyethanol appeared to increase with time (Table 5.3). The mean anaesthesia rates using 2-phenoxyethanol during the initial weighing and measuring of shell length were significantly shorter than during the final weighing and measuring exercise.

In the largest size class (60-90 mm SL), mean total anaesthesia rates were not significantly different from each other for the first four months. However, the mean total anaesthesia rates

Table 5.2. The mean total exposure time to $MgSO_4$, mean exposure time to air during weighing and measuring, and mean total recovery rates after replacement into anaesthetic-free seawater of three size classes of *Haliotis midae*. Standard deviations are presented. Different superscripts indicate significant differences in mean total anaesthesia rates, recovery rates and exposure to air at $p<0.05$.

$MgSO_4$						
Conc. g.100 ml^{-1}	Size (mm SL)	Month	Temp. (°C)	Mean total exposure time to anaesthetic (min)	Mean total exposure time to air (min)	Mean total recovery time (min)
4	5-15	1	18.5	1.74 ± 0.19^{a}	12.34 ± 0.93^{b}	9.12 ± 2.34^{c}
4		2	18.1	1.66 ± 0.50^{a}	12.00 ± 1.10^{b}	12.16 ± 2.64^{c}
4		3	19.0	1.60 ± 0.69^{a}	11.43 ± 0.92^{b}	10.72 ± 1.04^{c}
6*		4	16.9	1.11 ± 0.16^{a}	14.34 ± 2.98^{b}	9.13 ± 2.54^{c}
4		5	19.2	1.30 ± 0.39^{a}	10.57 ± 2.35^{b}	12.24 ± 1.86^{c}
6*		6	17.2	1.27 ± 0.09^{a}	10.17 ± 2.52^{b}	12.16 ± 3.93^{c}
4		7	19.5	1.22 ± 0.06^{a}	12.01 ± 1.68^{b}	11.37 ± 1.00^{c}
4		8	19.3	1.62 ± 0.52^{a}	9.74 ± 0.75^{b}	7.81 ± 1.02^{c}
14	20-50	1	18.5	5.28 ± 1.00^{d}	12.23 ± 1.01^{e}	18.25 ± 0.94^{f}
14		2	18.1	5.59 ± 0.56^{d}	12.30 ± 1.70^{e}	11.72 ± 2.85^{f}
14		3	19.0	6.56 ± 1.69^{d}	10.03 ± 0.14^{e}	11.61 ± 1.40^{f}
18*		4	16.9	9.75 ± 2.84^{d}	13.17 ± 4.71^{e}	22.18 ± 4.76^{f}
14		5	19.2	5.15 ± 2.48^{d}	13.64 ± 4.36^{e}	15.79 ± 4.26^{f}
18*		6	17.2	6.07 ± 1.56^{d}	12.50 ± 2.25^{e}	19.72 ± 1.51^{f}
14		7	19.5	5.51 ± 1.78^{d}	11.25 ± 0.85^{e}	13.45 ± 0.88^{f}
14		8	19.3	5.47 ± 0.31^{d}	10.15 ± 2.89^{e}	15.50 ± 8.42^{f}
24	60-90	1	18.5	19.70 ± 5.15^{h}	13.38 ± 0.54^{i}	42.16 ± 4.57^{j}
24		2	18.1	22.44 ± 5.45^{h}	11.93 ± 1.30^{i}	40.99 ± 5.67^{j}
24		3	19.0	19.68 ± 0.73^{h}	11.33 ± 0.74^{i}	41.35 ± 6.23^{j}
30*		4	16.9	13.59 ± 4.65^{gh}	10.22 ± 1.18^{i}	31.26 ± 18.67^{j}
24		5	19.2	15.81 ± 4.43^{gh}	10.00 ± 3.84^{i}	36.13 ± 13.17^{j}
30*		6	17.2	8.08 ± 1.02^{g}	9.77 ± 1.72^{i}	25.37 ± 14.46^{j}
24		7	19.5	14.96 ± 3.72^{gh}	12.24 ± 1.39^{i}	35.39 ± 6.94^{j}
24		8	19.3	17.53 ± 4.97^{gh}	10.69 ± 3.03^{i}	33.15 ± 11.47^{j}

* Higher concentrations used at lower temperatures.

during the last three months were significantly shorter than those during the first four months. Mean total exposure to air during weighing and measuring in animals treated with 2-phenoxyethanol did not differ significantly from each other except for the largest size class (60-90 mm SL). There were also no significant differences in the mean total recovery times for the 5-15 and 20-50 mm SL animals exposed to 2-phenoxyethanol anaesthesia. However, mean total recovery rates in the 60-90 mm SL size class appeared to become increasingly longer during the later months.

Table 5.3. The mean total exposure time to 2-phenoxyethanol, mean exposure time to air during weighing and measuring, and mean total recovery rates after replacement into anaesthetic-free seawater of three size classes of *Haliotis midae*. Standard deviations are presented. Different superscripts indicate significant differences in mean total anaesthesia rates, recovery rates and exposure to air at $p < 0.05$.

2-phenoxyethanol

Conc. g.100 ml^{-1}	Size (mm SL)	Month	Temp. (°C)	Mean total exposure time to anaesthetic (min)	Mean total exposure time to air (min)	Mean total recovery time (min)
0.05	5-15	1	18.5	1.43±0.11^{a}	11.73±2.62^{c}	32.89±9.49^{d}
		2	18.1	1.94±0.14^{ab}	12.50±0.65^{c}	31.13±7.54^{d}
		3	19.0	2.30±0.90^{ab}	10.75±1.45^{c}	31.89±2.44^{d}
		4	16.9	2.84±1.13^{ab}	11.87±2.00^{c}	38.46±8.27^{d}
		5	19.2	2.57±0.55^{ab}	10.09±0.83^{c}	33.54±6.66^{d}
		6	17.2	2.96±0.61^{ab}	9.57±0.49^{c}	32.64±7.21^{d}
		7	19.5	3.83±0.66^{b}	9.56±0.74^{c}	36.24±3.17^{d}
0.2	20-50	1	18.5	3.18±0.36^{e}	12.52±1.56^{g}	40.89±7.69^{h}
		2	18.1	4.38±1.44^{ef}	10.18±3.21^{g}	38.38±11.22^{h}
		3	19.0	5.14±0.45^{ef}	9.99±1.22^{g}	34.82±6.86^{h}
		4	16.9	6.79±1.46^{ef}	8.79±1.40^{g}	41.59±4.67^{h}
		5	19.2	7.01±1.99^{ef}	12.13±1.30^{g}	46.81±7.27^{h}
		6	17.2	7.58±2.58^{ef}	10.44±2.62^{g}	46.38±8.87^{h}
		7	19.5	9.45±0.51^{f}	9.18±1.62^{g}	50.64±1.10^{h}
0.3	60-90	1	18.5	7.43±1.37^{j}	13.05±0.78^{k}	25.09±1.48^{m}
		2	18.1	5.72±0.52^{ij}	10.99±1.34^{kl}	29.95±8.71^{m}
		3	19.0	8.52±1.42^{j}	10.98±1.37^{kl}	27.63±5.83^{m}
		4	16.9	9.85±4.09^{j}	7.79±4.03^{kl}	67.61±44.26
		5	19.2	2.34±0.81^{i}	7.65±5.50^{kl}	^{mn}
		6	17.2	1.86±0.39^{i}	1.95±0.13^{kl}	135.50±5.80^{n}
		7	19.5	2.92±0.12^{ij}	1.97±0.94^{l}	141.30±3.02^{n}
						151.05±1.07^{n}

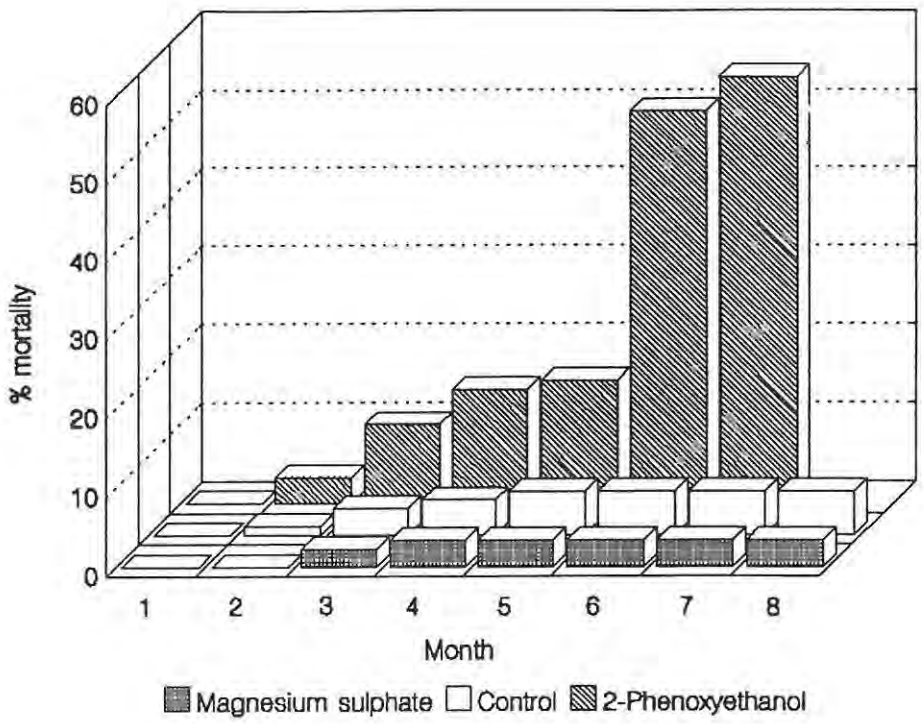


Figure 5.2. Percent accumulated monthly mortality of the 5-15 mm SL *Haliotis midae* exposed to $MgSO_4$ and 2-phenoxyethanol anaesthesia during an eight month growth trial.

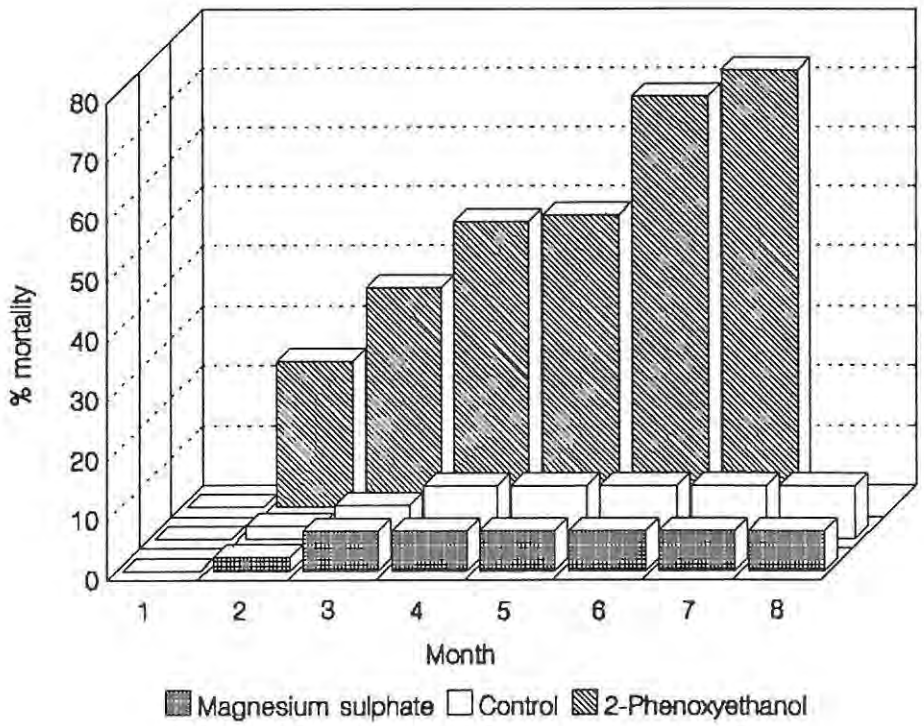


Figure 5.3. Percent accumulated monthly mortality of the 20-50 mm SL *Haliotis midae* exposed to $MgSO_4$ and 2-phenoxyethanol anaesthesia during an eight month growth trial.

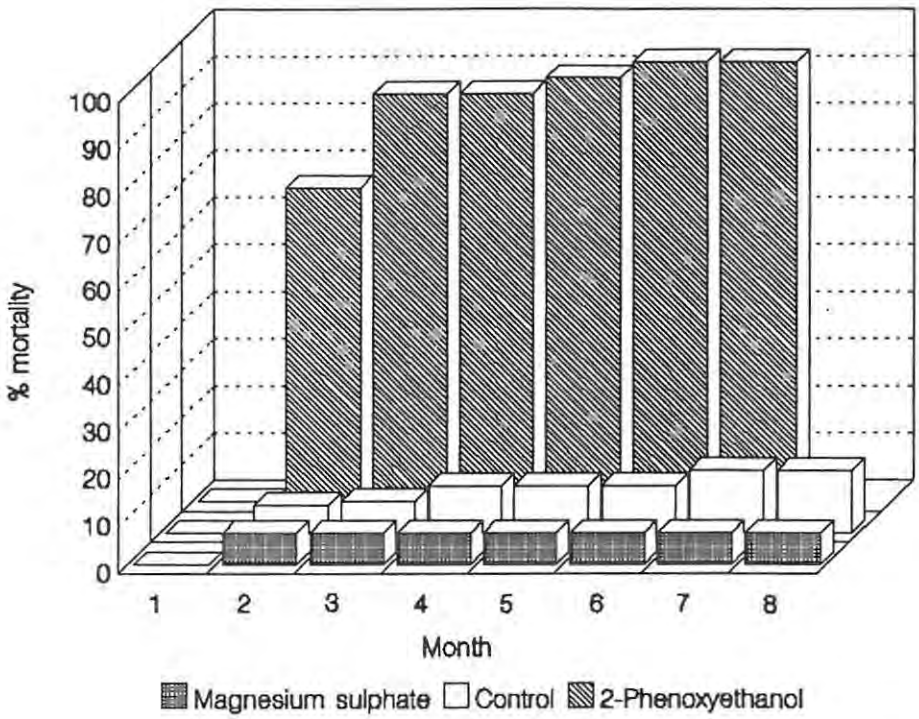


Figure 5.4. Percent accumulated monthly mortality of the 60-90 mm SL *Haliotis midae* exposed to $MgSO_4$ and 2-phenoxyethanol anaesthesia during an eight month growth trial.

The mortality of 2-phenoxyethanol treated animals was significantly greater than those in the control groups and those anaesthetized with $MgSO_4$ (Table 5.4 and Figures 5.2 to 5.4). In the 2-phenoxyethanol treated animals, a maximum percent mortality of 93.33% was recorded, compared to 13.33% and 6.67% for the control and the $MgSO_4$ treated animals, respectively.

Comparison of the slopes of regression models for monthly shell length and weight increases in the two smaller size classes (5-15 and 20-50 mm SL) showed that there were no significant differences in growth between the control group and $MgSO_4$ treated animals, except for the shell length increase in the 5-15 mm SL animals (Figures 5.5 to 5.12). The slope for the monthly increases in shell length in this size class was significantly higher in $MgSO_4$ treated animals ($F = 8.86$, $p<0.05$, $df = 1$, $n = 1395$). The slopes of the regression models for monthly increases in weight and shell length of untreated and $MgSO_4$ treated 60-90 mm SL animals could not be compared, even though there was a total increase in shell length and weight in both control and $MgSO_4$ treated animals (Table 5.5). This was due to large variations in shell length and weight within groups, as well as to the very slow growth rates in this size class.

Table 5.4. The percent accumulated monthly mortality of three size classes of untreated and regularly $MgSO_4$ and 2-phenoxyethanol anaesthetized *Haliotis midae*.

		Control group	$MgSO_4$ treated group	2-Phenoxyethanol treated group
Size (mm SL)	Month	Accumulated percent mortality	Accumulated percent mortality	Accumulated percent mortality
5-15	1	0	0	0
	2	1.11	0	3.33
	3	3.33	2.22	10.00
	4	4.44	3.33	14.44
	5	5.56	3.33	15.56
	6	5.56	3.33	50.00
	7	5.56	3.33	54.44
	8	5.56	3.33	*
20-50	1	0	0	0
	2	2.22	2.22	24.44
	3	5.56	6.67	36.67
	4	8.88	6.67	47.78
	5	8.88	6.67	48.89
	6	8.88	6.67	68.89
	7	8.88	6.67	73.33
	8	8.88	6.67	*
60-90	1	0	0	0
	2	6.67	6.67	66.67
	3	6.67	6.67	86.67
	4	10.00	6.67	86.67
	5	10.00	6.67	90.00
	6	10.00	6.67	93.33
	7	13.33	6.67	93.33
	8	13.33	6.67	*

* Growth trial discontinued due to high percent mortality in previous months.

DISCUSSION

The increase in mean anaesthesia rates each month in animals treated with 2-phenoxyethanol, was hypothesized not to be related to temperature, but rather due to the development of resistance to the efficacy of the anaesthetic. The increase in recovery rate in animals anaesthetized with 2-phenoxyethanol was probably due to the a combination of longer exposure times to the anaesthetic and stress. The latter was confirmed by the high percent mortality noted for the 2-phenoxyethanol treated animals each month.

In the preliminary trials described in Chapters 2 and 3, 2-phenoxyethanol appeared to have good abalone anaesthetic potential. However, the increased resistance to the efficacy of the

anaesthetic and the high percent mortalities recorded each month (up to 100% in some replicates) in this experiment, renders this anaesthetic unsuitable for abalone anaesthesia. 2-Phenoxyethanol was therefore not considered in any further investigations. $MgSO_4$, on the other hand, did not affect growth in the 5-15 and the 20-50 mm SL animals. Moreover, slightly lower mortalities (though not significant) were recorded in the $MgSO_4$ treated groups in comparison to the control groups. Moreover, given that the animals were anaesthetized at intervals shorter than would normally be the case under commercial culture conditions, suggests that $MgSO_4$ is very suitable for use as an anaesthetic in commercial abalone culture.

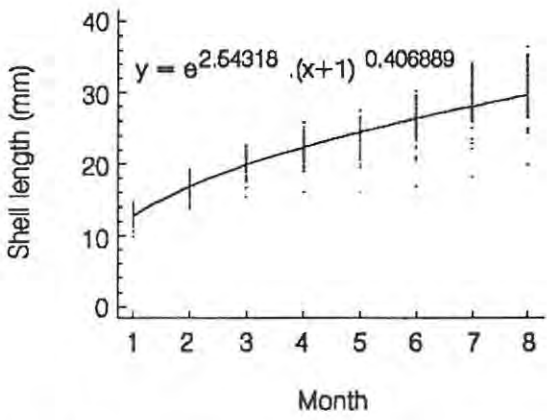


Figure 5.5. Increase in shell length of 5-15 mm SL *Haliotis midae* exposed to $MgSO_4$ anaesthesia on a monthly basis.

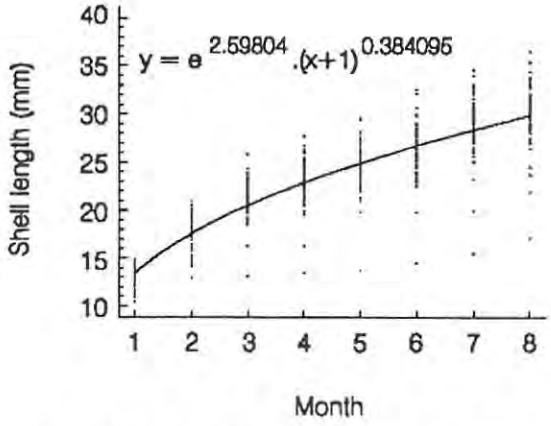


Figure 5.6. Monthly increase in shell length of untreated 5-15 mm SL *Haliotis midae*.

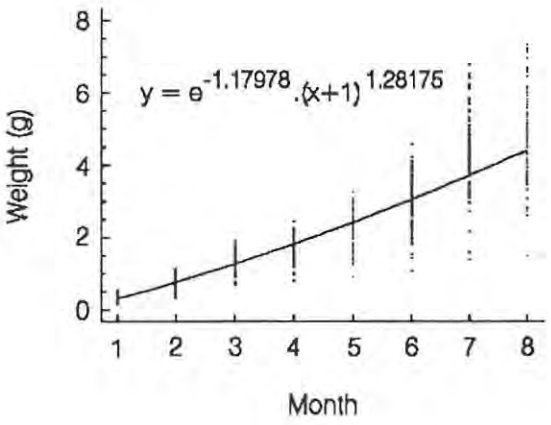


Figure 5.7. Increase in weight of 5-15 mm SL *Haliotis midae* exposed to $MgSO_4$ anaesthesia on a monthly basis.

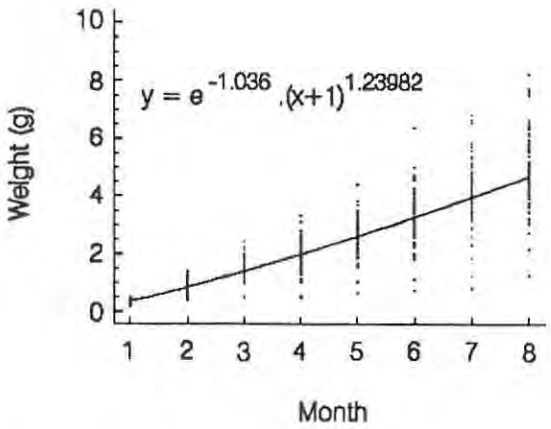


Figure 5.8. Monthly increase in weight of untreated 5-15 mm SL *Haliotis midae*.

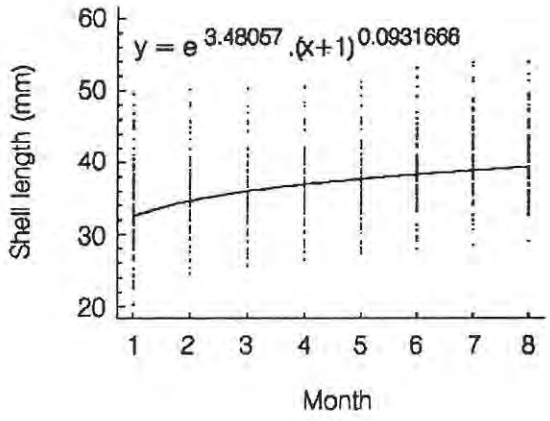


Figure 5.9. Increase in shell length of 20-50mm SL *Haliotis midae* exposed to $MgSO_4$ anaesthesia on a monthly basis.

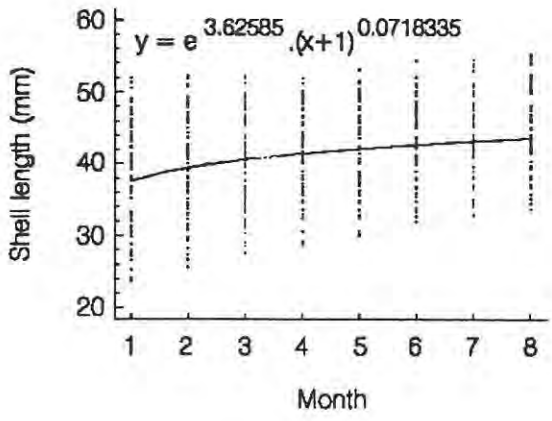


Figure 5.10. Monthly increase in shell length of untreated 20-50mm SL *Haliotis midae*.

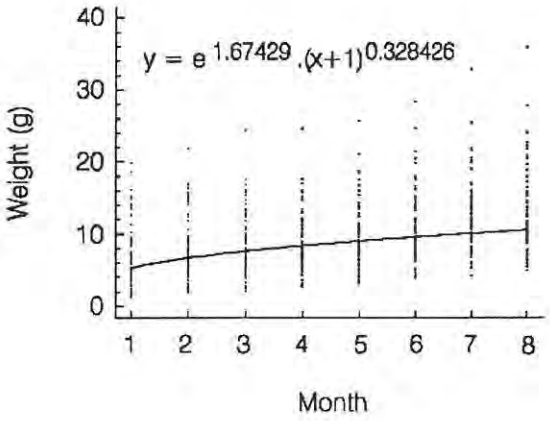


Figure 5.11. Increase in weight of 20-50 mm SL *Haliotis midae* exposed to $MgSO_4$ anaesthesia on a monthly basis.

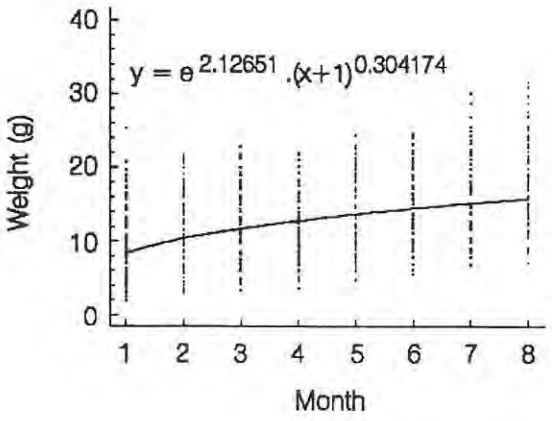


Figure 5.12. Monthly increase in weight of untreated 20-50 mm SL *Haliotis midae*.

However, other aspects need to be considered before any final conclusions regarding its suitability for commercial application can be made. It is very important that an anaesthetic should not affect flesh texture or leave residues, and it is also essential to establish the effect of prolonged exposure to anaesthesia. These aspects will be dealt with in the next three chapters.

Table 5.5. Initial, final and total increase in shell length and weight of untreated and regularly anaesthetized *Haliotis midae* after an eight month growth trial. Standard deviations of the means are also presented.

	Control group					
	Shell length (mm)			Weight (g)		
Size (mm SL)	Initial (range)	Final (range)	Increase	Initial (range)	Final (range)	Increase
5-15	13.15±1.02 (10.4-14.9)	30.08±2.83 (17.1-36.5)	16.94±1.81	0.35±0.08 (0.16-0.52)	4.93±1.21 (1.24-8.24)	4.58±1.13
20-50	38.90±7.53 (23.7-51.9)	44.96±5.73 (33.7-55.2)	6.06±1.80	10.44±5.45 (1.94-25.33)	18.39±6.16 (7.07-31.58)	7.96±0.71
60-90	71.89±8.09 (58.0-87.4)	75.50±8.53 (61.8-92.1)	3.61±0.43	63.13±21.12 (30.9-104.39)	78.61±25.13 (42.03-133.28)	15.45±4.01
	$MgSO_4$ treated group					
5-15	12.82±0.97 (9.9-14.7)	30.42±2.73 (19.9-36.5)	17.60±1.76	0.34±0.08 (0.13-0.53)	4.78±1.18 (1.49-7.34)	4.44±1.10
20-50	33.55±6.19 (20.3-49.5)	40.80±5.06 (29.1-54.0)	7.25±1.12	6.91±4.03 (1.32-19.8)	12.75±5.32 (4.94-35.91)	5.84±1.29
60-90	63.67±6.57 (55.9-78.3)	69.88±6.55 (57.8-86.2)	6.20±0.01	45.27±15.71 (23.2-84.3)	60.52±17.39 (32.79-106.34)	15.25±1.68

CHAPTER 6

EFFECT OF MAGNESIUM SULPHATE ANAESTHESIA ON ABALONE MUSCLE ULTRASTRUCTURE

INTRODUCTION

The texture of abalone meat is related to the distribution of proteins within the foot (Olley & Thrower 1977). The pedal sole is rich in collagen (Voltzow 1990, Frescura 1990), which renders it noticeably tough compared to the columellar muscle (Olley & Thrower 1977). Another factor which may influence the texture of abalone meat is the treatment to which the live animal has been subjected. In this respect it is important that magnesium sulphate ($MgSO_4$) anaesthesia should not affect the ultra-structure of the tarsal or columellar muscle, since this might effect texture which in turn might affect marketability.

As mentioned in Chapter 2, the musculature of the abalone foot is divided into two functionally and structurally distinct regions: the tarsal and the columellar muscle (Fretter & Graham 1962, Trueman & Brown 1985, Frescura 1990). The columellar muscle is responsible the major body movements and changes in shape and posture: protraction, retraction, twisting, elevation and lowering of the shell and clamping down onto the substratum. The tarsal muscle also shares responsibility for adherence onto substratum and is involved in the fine movements of locomotion and food manipulation.

Frescura (1990) and Voltzow (1990) studied the fine structure of *Haliotis spadicea* and *H. kamtschatkana* pedal musculature, respectively. Both these authors found that the tarsal muscle contains finer branched muscle bundles and a larger proportion of collagenous connective tissue than the columellar muscle. The columellar muscle consists of bundles of muscle fibres wrapped in thin connective tissue sheaths. Virtually no other extracellular connective tissue is present. In the tarsic region the connective-tissue sheaths become thicker. The bundles of muscle fibres in the tarsic region branch and change direction as they extend from their origin to their insertions. The bundles become finer as they approach the periphery of the foot and become more deeply embedded in the connective tissue of the ventral and lateral extremities. The tarsal muscle also contains larger numbers of mitochondria than the columellar muscle.

Frescura (1990) measured muscle cell, thick filament, thin filament and mitochondrion diameter of the columellar muscle of *H. spadicea* and found it to range between 3 and 6 μm, 33-75 nm, 6-7 nm and 0.5-0.9 μm respectively. No measurements of tarsal muscle thick filament, thin filament or mitochondrion diameter were recorded by Frescura (1990).

The aim of this investigation was to compare the fine structure of both tarsal and columellar muscle tissue of untreated *H. midae* and animals which had regularly been subjected to $MgSO_4$ anaesthesia, on the assumption that such changes would be indicative of changes in texture.

MATERIALS AND METHODS

Columellar and tarsal muscle samples were taken from a control group of untreated animals (n = 3) and from animals which had been exposed to monthly $MgSO_4$ anaesthesia over a eight month period (n = 3). Tissue samples were prepared for transmission electron microscopy using the following protocol (Cross 1985). The musculature of live *H. midae* (70-80 mm shell length (SL)) was flooded with cold 2.5 % glutaraldehyde made up in 0.2 M sodium cacodylate buffer after which pieces of tissue about 2-3 mm^3 were excised from the centre of the columellar muscle and tarsal muscle. The tissue samples were then placed in fresh buffered glutaraldehyde and cut into smaller pieces of about 1 mm^3. Primary fixation in 2.5 % buffered glutaraldehyde was allowed to proceed for 12 hours at 14°C. The tissue samples were then rinsed twice for 10 min in cacodylate buffer, followed by secondary fixation in 1 % osmium tetroxide for 90 min. Following secondary fixation the tissue was rinsed once more in the cold buffer for 10 min and then dehydrated for 5 min in each of a 30, 50, 70, 80 and 90 % ethanol series, and twice in absolute ethanol. After dehydration, the tissue was embedded via propylene oxide in an Araldite CY 212/Taab 812 resin mixture which was polymerized for 36 hours at 60°C. Sections showing gold interference colour (60-70 nm) were cut using glass knives and stained at room temperature with aqueous uranyl acetate solution (5 %) for 30 min, followed by Reynold's lead citrate solution for 10 min. The sections were viewed using a JEOL JEM 100 CXII transmission electron microscope.

Muscle cell diameter (n = 40), thick filament diameter (n = 600) and thin filament diameter (n = 400) in columellar and tarsal muscle of treated and untreated animals were measured and compared to determine any structural changes in animals treated with $MgSO_4$. Peripheral mitochondrion lengths (n=40) in both types of muscle in the control and $MgSO_4$ treated

group were also compared. The number of thick and thin filaments per μm^2 (n = 40) in both columellar and tarsal muscle of untreated and treated animals were also compared.

Statistical analysis

The diameter of muscle cells, thick filaments and thin filaments, and mitochondria length in both tarsal and columellar muscle of treated and untreated animals were tested for significant differences using the Student T-test ($p \leq 0.05$). The test was also used to determine significant differences in the number of thin and thick filaments in tarsal and columellar muscle of treated and untreated *H. midae* (Zar 1984).

RESULTS

Muscle cell diameter in columellar muscle of the control group and $MgSO_4$ treated animals ranged between 3.1 and 6.21 μm^2, and 3.28 and 6.55 μm^2 respectively (Table 6.1). Cell diameter of the tarsal muscle sections ranged between 1.11 and 4.28 μm^2 in the control group, and between 1.03 and 4.14 μm^2 in $MgSO_4$ treated animals. All filament diameters were measured in transverse sections. Thick filament diameter of columellar muscle in untreated and $MgSO_4$ treated animals ranged from 30 to 75 nm in both groups (Table 6.1, Figures 6.1 to 6.3). Thick filament diameter of tarsal muscle ranged from 25 to 60 nm in both groups (Table 6.1, Figures 6.4 to 6.6). Thin filament diameter of tarsal and columellar muscle ranged from 5.26 to 7.9 nm in both groups (Table 6.1). Filament lengths were not compared, since these were very difficult to measure as they meandered in and out of the plane of the sections.

Mitochondria were very scarce and only found at the periphery of cells and not centrally. This was also observed by Frescura (1990) for *H. spadicea*. Peripheral mitochondria of columellar muscle in the control and $MgSO_4$ treated group ranged from 0.45 to 1.2 μm and from 0.5 to 1.2 μm in length, respectively (Table 6.1). Peripheral mitochondria of tarsal muscle were between 0.5 and 1.3 μm long in the control group and between 0.5 and 1.25 μm long in $MgSO_4$ treated animals (Table 6.1, Figures 6.4 & 6.6).

The number of thick filaments in columellar muscle varied between 23 and 29 per μm^2 in the control group and between 23 and 30 per μm^2 in $MgSO_4$ treated animals (Table 6.1). In tarsal muscle, the number of thick filaments varied between 30 and 43 per μm^2 in the control

Table 6.1. Comparison of morphological features of columellar and tarsal muscle in untreated *Haliotis midae* and *Haliotis midae* treated with $MgSO_4$.

Treatment	Muscle	D_M (μm)	Range (μm)	D_T (nm)	Range (nm)	D_t (nm)	Range (nm)	M (μm)	Range (μm)	N_T	Range	N_t	Range
Control	Columellar	4.63	3.1-6.21	56.33	30-75	6.59	5.26-7.9	0.82	0.45-1.2	26	23-29	596	521-706
$MgSO_4$	Columellar	4.66	3.28-6.55	56.27	30-75	6.73	5.26-7.9	0.88	0.5-1.2	26	23-30	609	505-690
Control	Tarsal	2.60	1.11-4.28	42.75	25-60	6.16	5.26-7.9	0.81	0.5-1.3	37	30-43	627	521-724
$MgSO_4$	Tarsal	2.38	1.03-4.14	42.37	25-60	6.17	5.26-7.9	0.84	0.5-1.25	38	32-44	631	505-732

D_M = mean diameter of muscle cells (n = 40)
D_T = mean diameter of thick filaments (n = 600)
D_t = mean diameter of thin filaments (n = 400)
M = mean diameter of peripheral mitochondria (n = 40)
N_T = number of thick filaments per μm^2 area (n = 40)
N_t = number of thin filaments per μm^2 area (n = 40)

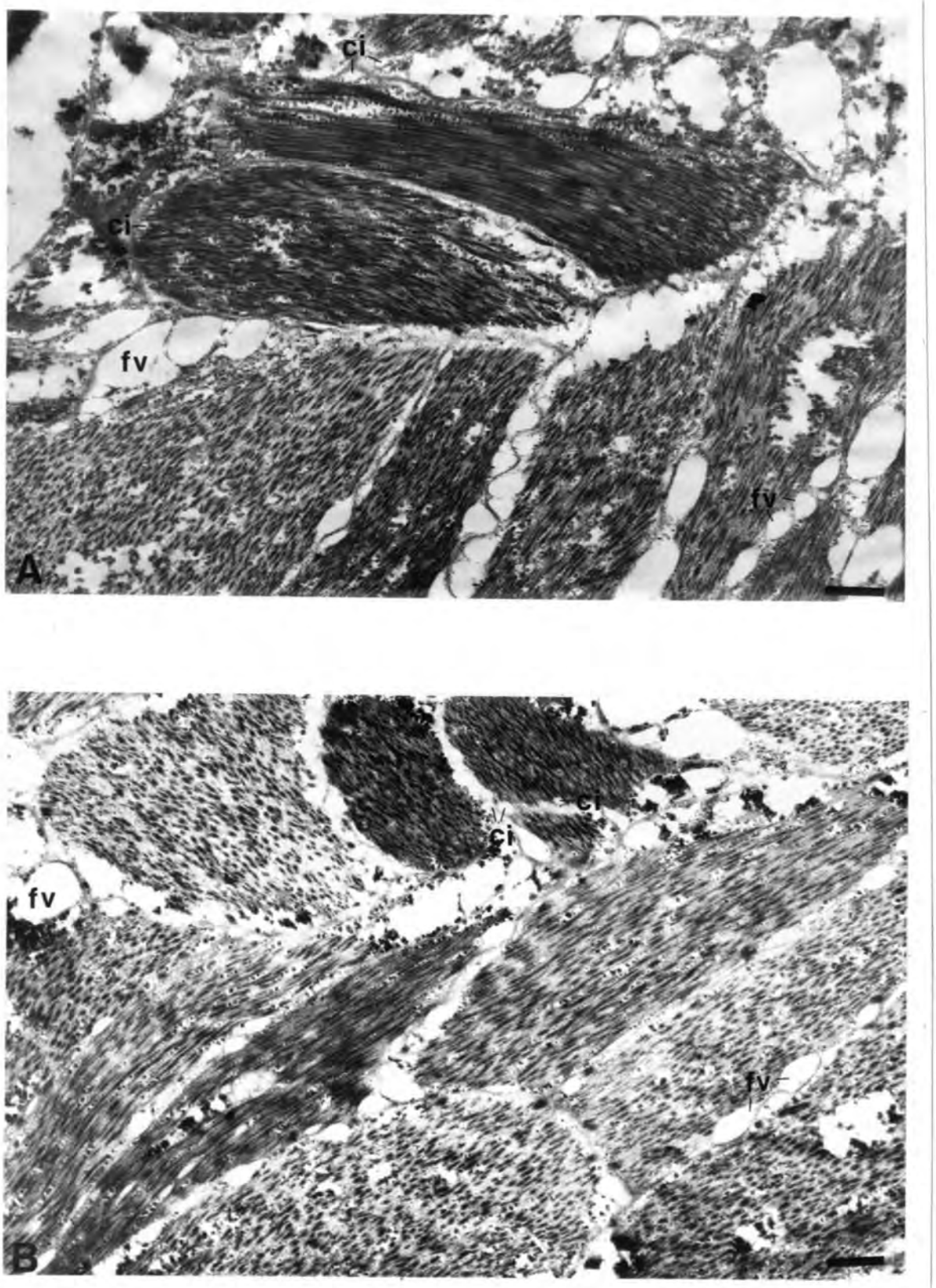


Figure 6.1. Electron micrographs of transverse and longitudinal sections of *Haliotis midae* columellar muscle. A. Untreated at a magnification of X9 280. Scale bar = 1 μm. B. $MgSO_4$ treated at a magnification of x9 280. Scale bar = 1 μm. Note the subsarcolemmal cisternae (ci) and fluid vesicles (fv).

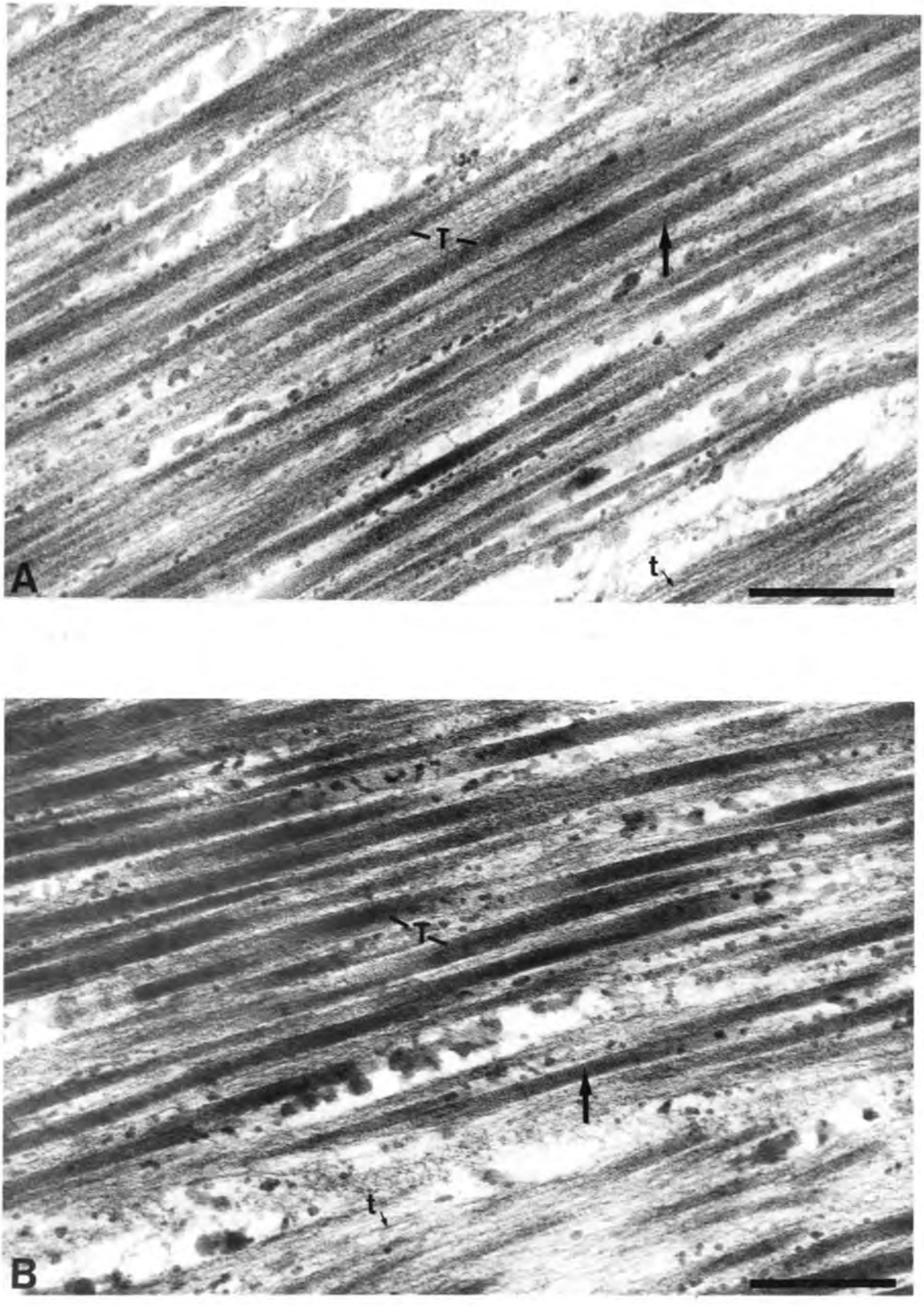


Figure 6.2. Electron micrographs of longitudinal sections of *Haliotis midae* columellar muscle. A. Untreated at a magnification of x46 400. Scale bar = 0.5 μm. B. $MgSO_4$ treated at magnification of x46 400. Scale bar = 0.5 μm. Note the axial striations (arrow), thick filaments (T) and thin filaments (t).

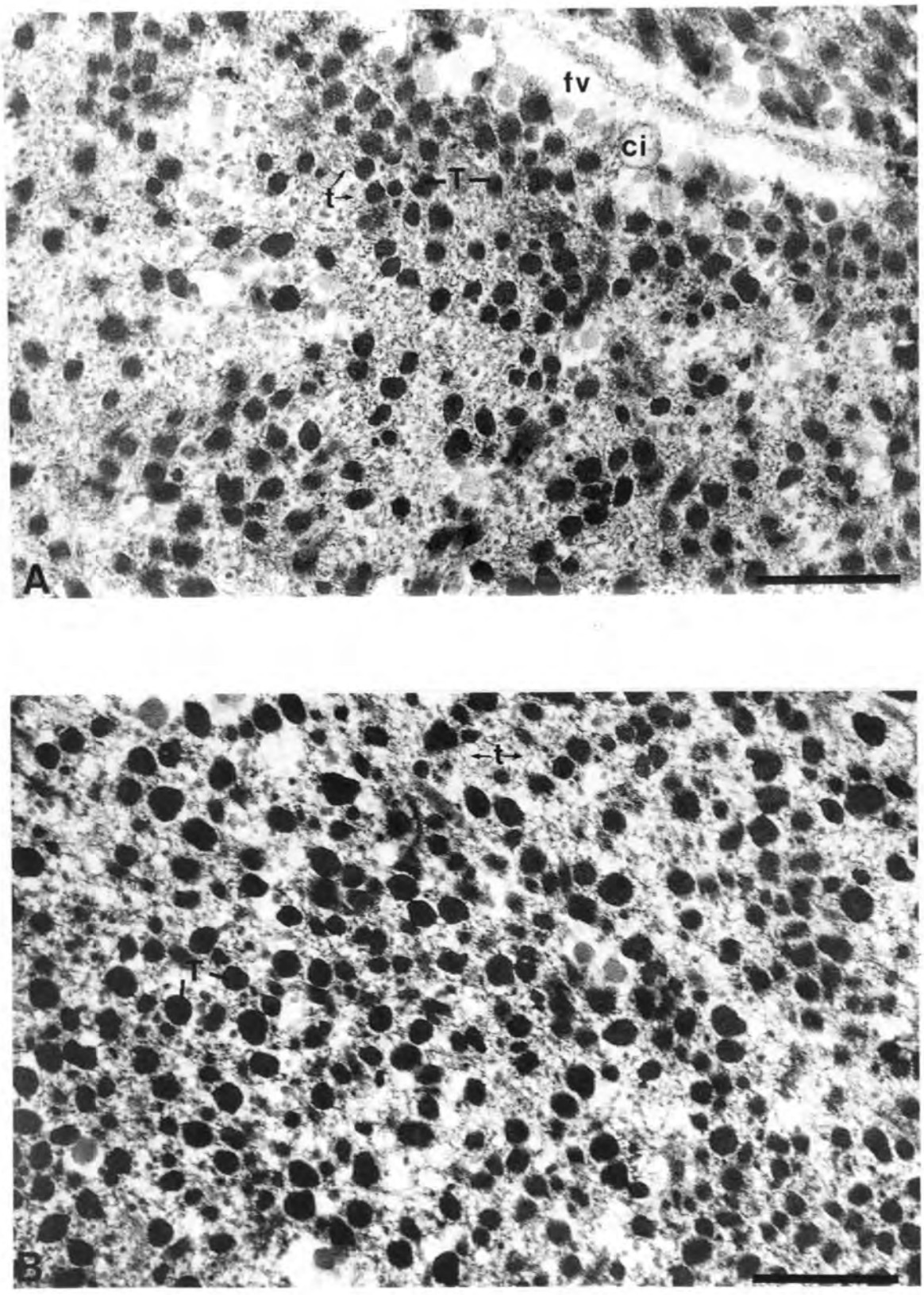


Figure 6.3. Electron micrographs of transverse sections of *Haliotis midae* columellar muscle. A. Untreated at a magnification of x46 400. Scale bar = 0.5 μm. B. $MgSO_4$ treated at a magnification of x46 400. Scale bar = 0.5 μm. Note the subsarcolemmal cisternae (ci), fluid vesicles (fv), thick filaments (T) and thin filaments (t).

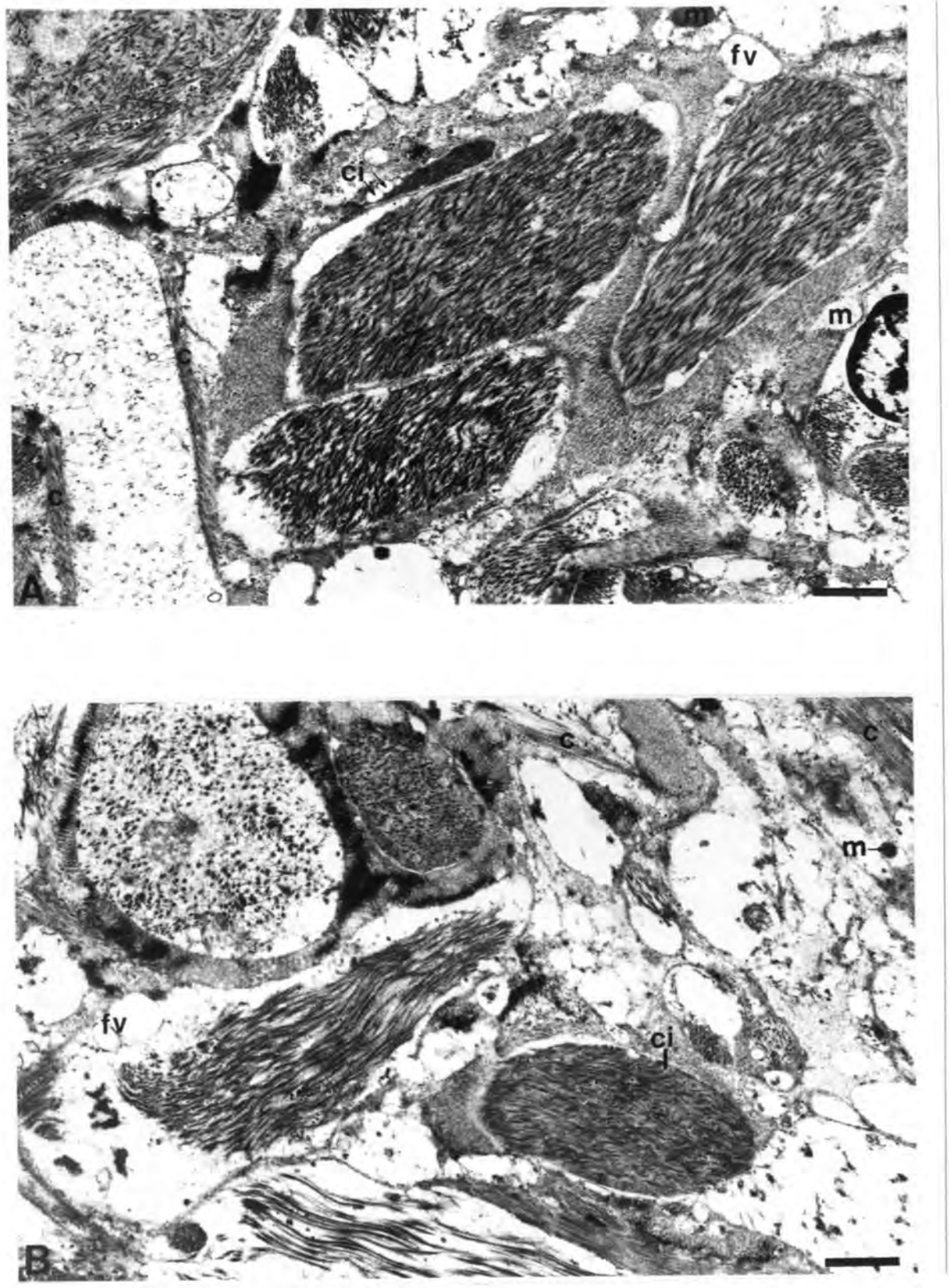


Figure 6.4. Electron micrographs of transverse and longitudinal sections of *Haliotis midae* tarsal muscle. A. Untreated at a magnification of x11 520. Scale bar = 1.0 μm. B. $MgSO_4$ treated at a magnification of x11 520. Scale bar = 1 μm. Note the collagen (c), subsarcolemmal cisternae (ci), fluid vesicles (fv) and peripheral mitochondria (m).

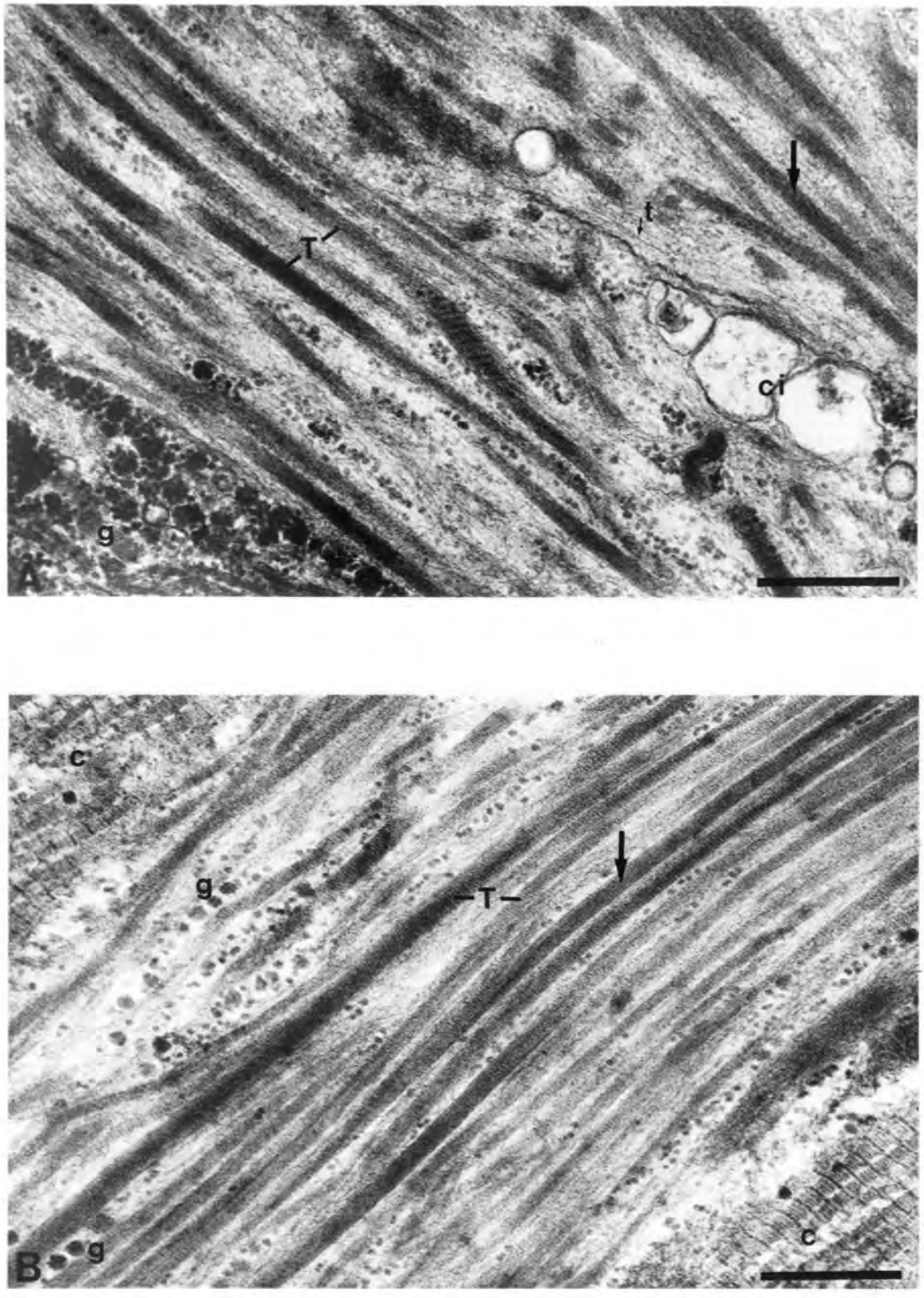


Figure 6.5. Electron micrographs of longitudinal sections of *Haliotis midae* tarsal muscle . A. Untreated at a magnification of x46 400. Scale bar = 0.5 μm. B. $MgSO_4$ treated at a magnification of x46 400. Scale bar = 0.5 μm. Note the axial striations (arrow), collagen (c), subsarcolemmal cisternae (ci), glycogen granules (g), thick filaments (T) and thin filaments (t).

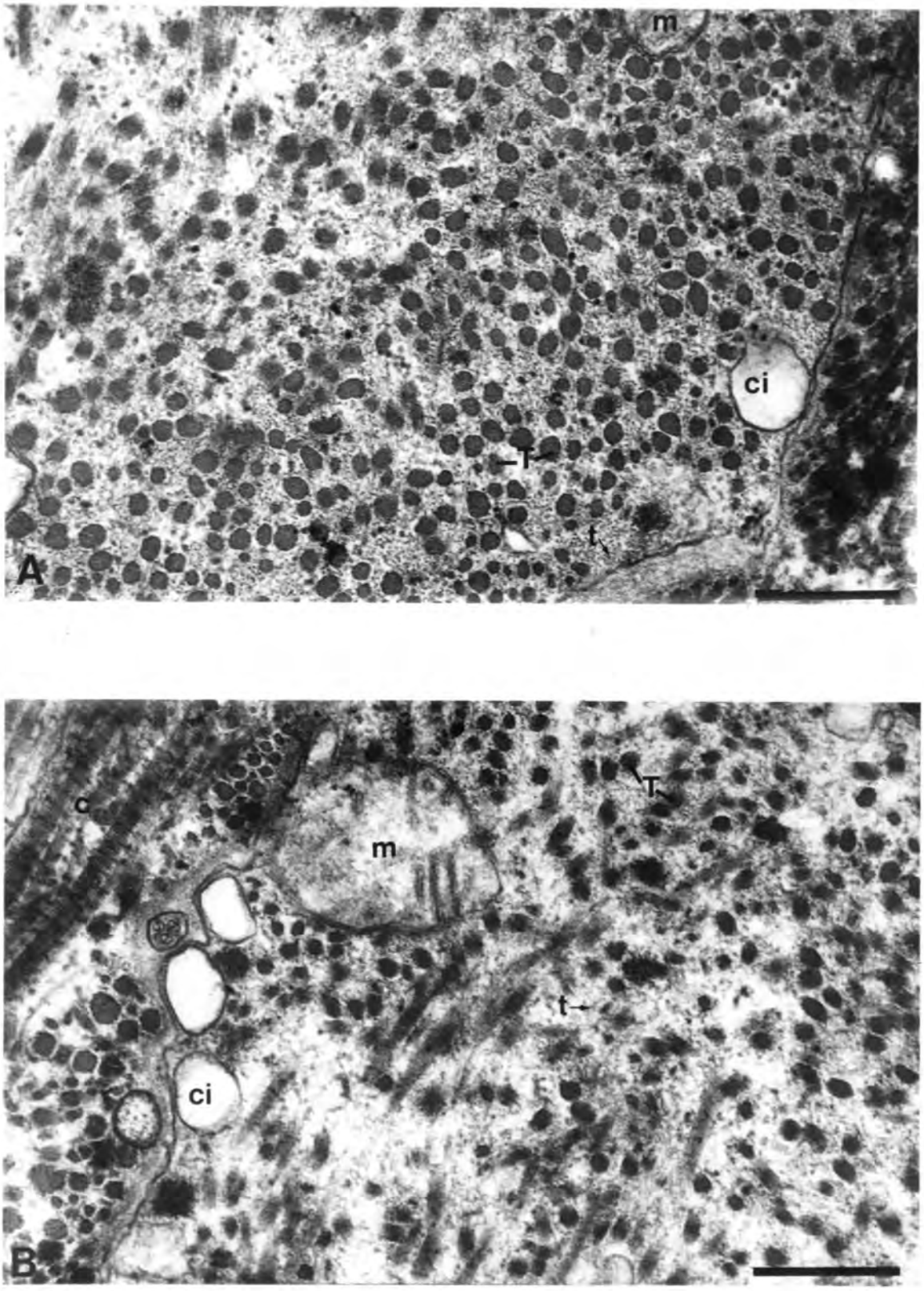


Figure 6.6. Electron micrographs of transverse sections of *Haliotis midae* tarsal muscle. A. Untreated at a magnification of x46 400. Scale bar = 0.5 μm. B. $MgSO_4$ treated at a magnification of x46 400. Scale bar = 0.5 μm. Note the collagen (c), subsarcolemmal cisternae (ci), peripheral mitochondrion (m), thick filaments (T) and thin filaments (t).

group and between 32 and 44 per μm^2 in $MgSO_4$ treated animals. The number of thin filaments in columellar muscle varied between 521 and 706 per μm^2 in the control group and between 505 and 690 per μm^2 in $MgSO_4$ treated animals. In tarsal muscle, it varied between 521 and 724 per μm^2 in the control group and between 505 and 732 per μm^2 in $MgSO_4$ treated animals.

There were no significant differences in muscle cell diameter, thick and thin filament diameter, mitochondrion length, or the number of thick and thin filaments per μm^2 in columellar and tarsal muscle between the control group and $MgSO_4$ treated animals.

DISCUSSION

The mean muscle cell diameters of columellar muscle in the control group and $MgSO_4$ treated animals were 4.63 and 4.66 μm^2 respectively; and for tarsal muscle, 2.60 and 2.38 μm^2 respectively. The mean thick filament diameter of columellar muscle in the control group and $MgSO_4$ treated animals was 56.33 and 56.27 nm respectively, and the mean thick filament diameter of tarsal muscle was 42.75 nm in the control group and 42.37 nm in $MgSO_4$ treated animals.

The smaller diameter of muscle cells and of thick filaments in the tarsal muscle ties in with the suggestion that it possibly increases the agility and rate of contraction of this muscle in comparison to the columellar muscle (Frescura 1990, and see Chapter 2). Frescura (1990) also found that the diameter of the thick filaments of the tarsal muscle of the limpet, *Patella oculus* is smaller than that of the thick filaments of columellar muscle. The thick filament diameter ranges in the columellar muscle of *H. midae* are similar to those in *H. spadicea* (Frescura 1990), viz. between 30 and 75 nm for *H. midae* and between 33 and 75 nm for *H. spadicea*. Frescura (1990) found that the diameter of thin filaments of columellar muscle in *H. spadicea* ranged between 6 and 7 nm. The diameter of thin filaments in *H. midae* columellar muscle ranged between 5.26 and 7.9 nm which was also similar to the results obtained by Frescura (1990). The length of the mitochondria in *H. midae* columellar muscle is also similar to those of *H. spadicea* (Frescura 1990), i.e. between 0.45 and 1.2 μm^2 in *H. midae* and between 0.5 and 0.9 μm^2 in *H. spadicea*.

There were no significant differences in mean muscle cell, thick filament and thin filament diameter, mitochondria length or the number of thin and thick filaments per μm^2 area in

columellar and tarsal muscle of the control group and the $MgSO_4$ treated animals. These results suggest that there was no modification of muscle tissue in animals treated with $MgSO_4$.

In conclusion, $MgSO_4$ has no effect on the ultrastructure and therefore, by implication, on the texture of *H. midae* muscle tissue, and can therefore be used for anaesthesia without any fear of affecting marketability. It is necessary however to still determine the relationship between muscle fine structure and texture before this conclusion can be finally accepted.

CHAPTER 7

ANAESTHETIC RESIDUES IN *HALIOTIS MIDAE* MUSCLE TISSUE AFTER SHORT TERM AND INTERMITTENT LONG TERM EXPOSURE TO\ MAGNESIUM SULPHATE

INTRODUCTION

Restrictions on the use of anaesthetics in fishery operations relate not only to the effectiveness or ineffectiveness of the chemicals, but also to the legality of their use in food fish (Marking & Meyer 1985). In the development of an anaesthetic, finding a compound that is effective is relatively easy in comparison to meeting the requirements of the U.S.A. Food and Drug Administration (FDA) for registration. The FDA requires that a drug used in aquaculture should be safe and effective for its intended use (Stefan 1992). This includes safety to the animal being anaesthetized, the person administering the drug, the consumer of the product and safety to the environment.

Since most anaesthetics are absorbed through the gills, residues in the tissues are likely to result unless adequate depuration time is allowed. The FDA requires that any compound used to anaesthetize fish that may be used for human consumption must be determined to be safe or inactive, if at all present by the time it reaches the consumer.

Methanesulfonate (MS-222) and carbon dioxide (CO_2) are the only anaesthetics registered for use on food fish (Schnick *et al.* 1979, Stefan 1992). Restrictions allow concentrations of 15-66 ppm MS-222 for 6-48 h and 50-330 ppm for 1-40 min for sedation and anaesthesia respectively, and require a 21-day depuration period (Schnick *et al.* 1979, Marking & Meyer 1985). CO_2 gas and sodium bicarbonate (142 to 642 ppm for 5 min as a means of introducing CO_2 into the water) are also allowed in aquaculture for anaesthetic purposes (Stefan 1992). CO_2 leaves no residues in animal tissue and does therefore not require a depuration period (Gilderhus & Marking 1987).

Tests for residues of anaesthetics in aquatic organisms documented to date are limited to two anaesthetics. These include residues of MS-222 in four salmonids (Walker & Schoettger 1967a, 1967b) and channel catfish, *Ictalurus punctatus* (Schoettger *et al.* 1967), and residues of benzocaine in rainbow trout, *Salmo gairdneri* (= *Oncorhynchus mykiss*), and large mouth

bass, *Micropterus salmoides* (Allen 1988).

Since muscle is the principle edible tissue in abalone, the aim of this investigation was to test abalone flesh for residues following magnesium sulphate ($MgSO_4$) anaesthesia. This would provide an indication of its safety for human consumption and whether a depuration period is required.

MATERIALS AND METHODS

Abalone ranging from 60-90 mm shell length (SL) were selected for this study. Animals were collected along the Eastern Cape Coast and allowed to acclimatize to laboratory conditions for a period of one month in our Port Alfred laboratory. Three groups of animals were used for this study: a control group of untreated abalone (n = 3), a group (n = 3) of animals prepared for analysis immediately after exposure to 30 g.100 ml^{-1} $MgSO_4$ for 20 min at 18°C (Treatment 1) and a group of animals (n = 3) which had been exposed to $MgSO_4$ anaesthesia at regular intervals during an eight month growth trial (Treatment 2). The following protocol was followed to analyze magnesium levels in columellar and tarsal muscle tissue. Samples of columellar muscle and tarsal muscle (1 cm x 1 cm) of each group were sectioned and placed in distilled water. Muscle tissue samples of animals from the growth trial group were sectioned 24 hours after the last exposure to $MgSO_4$ anaesthesia. Each of the tissue samples were weighed and digested in 1 ml of concentrated nitric acid (55%) at 80°C for two hours after which the solutions were diluted to 5 ml with distilled water. The diluted solutions were centrifuged at 3000 g for 10 min, the supernatant was poured off and stored in test tubes covered with parafilm at 4°C. Magnesium levels in the muscle tissue samples were analyzed using atomic absorbtion spectroscopy (Varian Atomic Absorption Spectrophotometer, AA/1275 Series).

Statistical Analysis

The levels of magnesium measured in columellar and tarsal muscle of treated and untreated animals were subjected to a One-Way Analysis of Variance. Means were compared using Tukey's Multiple Range Test at 5% error probability. Homogeneity of variances were tested with Bartlett's Test (Zar 1984).

RESULTS

One-Way Analysis of Variance showed that there were no significant differences in magnesium levels in muscle tissue between the control group and the treated groups ($p > 0.05$) (Table 7.1). There were also no significant differences in magnesium levels in tarsal and columellar muscle sections in the two treated groups and the control group.

Table 7.1. Magnesium levels in 60-90 mm SL *Haliotis midae* columellar and tarsal muscle tissue in a control group, animals exposed to 30 g.100 ml^{-1} $MgSO_4$ at 18°C with no depuration time (Treatment 1) and animals subjected to monthly $MgSO_4$ anaesthesia over eight months with a 24 hour depuration period (Treatment 2). Standard deviations of the means are presented.

Group	Muscle	Number of *H. midae*	Depuration (h)	Mean magnesium levels (ppm)
Control	Columellar	3	Control	170.80±59.15
Treatment 1	Columellar	3	0	162.67±54.83
Treatment 2	Columellar	3	24	254.72±46.34
Control	Tarsal	3	Control	199.38±40.51
Treatment 1	Tarsal	3	0	209.11±52.69
Treatment 2	Tarsal	3	24	168.99±39.22

DISCUSSION

The levels of magnesium in both columellar and tarsal muscle tissue of *H. midae* intermittently exposed to $MgSO_4$ over an eight month period, after a depuration period of 24 hours, and animals exposed to $MgSO_4$ immediately prior to analysis, approached those of background magnesium levels in the control group. These results suggest that $MgSO_4$ does not leave any residues in tarsal or columellar muscle tissue of *H. midae*, even when animals are anaesthetized with 30 g.100 ml^{-1} $MgSO_4$ for 20 min immediately prior to analysis.

In conclusion, $MgSO_4$ is recommended for use as an abalone anaesthetic since it leaves no residues in the muscle tissue, requires no depuration period for elimination of residues and is therefore safe for human consumption. Moreover, $MgSO_4$ is used in the food industry and for medicinal purposes.

CHAPTER 8
PROLONGED EXPOSURE OF *HALIOTIS MIDAE* TO MAGNESIUM SULPHATE ANAESTHESIA

INTRODUCTION

A prerequisite of the U.S.A. Food and Drug Administration (FDA) is that a drug used in aquaculture should be safe to the animals (Stefan 1992). Mortality-free anaesthesia is also an important financial consideration in aquaculture. It is therefore important to know how long animals can be exposed to an anaesthetic without any mortalities, and whether the animals can survive the anaesthetic exposure times required during commercial farming practices.

Sagara and Ninomiya (1970) revealed that ethyl carbamic acid (0.5 and 1%) did not adversely affect juvenile abalone, *Haliotis gigantea*, when they remained in these concentrations for up to 24 hours. However, when exposed to a 20% and 30% magnesium sulphate ($MgSO_4$) solution they started dying 3 and 2 hours after exposure, respectively. Exposure for 10 minutes or longer to a 1% chloral hydrate solution was also lethal to juvenile *H. gigantea*.

The aim of this investigation was to evaluate the effect of prolonged exposure of three size classes of *H. midae* to $MgSO_4$ anaesthesia.

MATERIALS AND METHODS

Three size classes of *H. midae* were used for this study. The small animals (5-15 mm shell length (SL)) were obtained from the Sea Plant Products hatchery in Hermanus, while larger animals (20-50 and 60-90 mm SL) were collected along the Eastern Cape Coast. Prior to the initiation of the experiments, the animals were acclimated to laboratory conditions for a period of one month. During the acclimation period, the animals were kept at a temperature of 18°C and a salinity of 35 ppt. All experiments were conducted at this temperature and salinity.

In the first experiment the abalone from all three size classes were exposed to $MgSO_4$ anaesthesia for 20 and 40 minutes. The concentrations of $MgSO_4$ used for the three size classes (5-15, 20-50 and 60-90 mm SL) were 4, 14 and 24 g.100 ml^{-1} $MgSO_4$, respectively. These concentrations were selected from Table 3.1 and were the same as those used during the growth trial described in Chapter 6.

Each trial was undertaken in triplicate in plastic buckets containing 5 l of continuously aerated seawater. Ten animals were placed in each bucket and allowed to attach to the sides. Once all the animals were firmly attached to the substratum, the seawater was poured out and replaced with the anaesthetic solution. The animals were exposed to the anaesthetic solution for the desired time period, after which anaesthesia was terminated by transferring the animals to a bucket containing fresh, aerated seawater. The animals were placed upside down on the bottom of the container and the time taken for each animal to recover was noted. Recovery was defined to be complete when the abalone turned right side up. Daily mortalities were recorded for two months after exposure to the anaesthetic.

In the second experiment the medium size abalone (20-50 mm SL) were exposed to 14 g.100 ml^{-1} $MgSO_4$ for 1, 2, 4, 6 and 8 hours. The same protocol as described above was followed in this experiment. Three replicates of ten animals were used in each treatment. The animals were monitored for two months after exposure to the anaesthetic and daily mortalities were recorded.

Statistical analysis

To test the effect of prolonged exposure on recovery rate, the data were subjected to One-Way Analysis of Variance, with the main effect being exposure time. Means were compared using Tukey's Multiple Range Test at 5% error probability. Homogeneity of variances were tested with Bartlett's Test (Zar 1984).

RESULTS

All the abalone in the three size classes exposed to their respective $MgSO_4$ concentrations for 20 and 40 minutes recovered once they were placed in fresh seawater. However, One-Way Analysis of Variance showed that recovery rates increased significantly with an increase in exposure time to the anaesthetic (Table 8.1 & 8.2). These trends were especially evident in the 20-50 mm SL size class (Table 8.2). The recovery rates of larger animals were also significantly longer than the recovery rates of smaller animals (Table 8.1).

No mortalities were recorded in any of the size classes exposed to $MgSO_4$ anaesthesia for 20 or 40 minutes (Table 8.1). Similarly, no mortalities were recorded in the 20-50 mm SL size class which were anaesthetized with the 14 g.100 ml^{-1} $MgSO_4$ solution after exposure times of 1 to 6 hours (Table 8.2). However, after an exposure time of 8 hours, 6.67% post-

recovery mortalities were recorded.

DISCUSSION

Prolonged exposure of 5-15, 20-50 and 60-90 mm SL *H. midae* to 4, 14 and 24 g.100 ml^{-1} $MgSO_4$ respectively, showed that these animals can be exposed to the anaesthetic for up to 40 minutes without any concern of post-recovery mortalities. The increased recovery rates at longer exposure times have no implications for the commercial abalone farmer. The important factor that should be taken into consideration is mortality-free anaesthesia rather than recovery time.

Table 8.1. The mean recovery rates, percentage recovery and percentage survival of 5-15, 20-50 and 60-90 mm SL *Haliotis midae* after prolonged exposure to 4, 14 and 24 g.100 ml^{-1} $MgSO_4$. Standard deviations of the means are presented. Different superscripts indicate significant differences in recovery rates at $p<0.05$.

Size (mm SL)	Conc. (g.100 ml^{-1})	Exposure time (min)	Mean recovery rate (min)	Percentage recovery	Percentage survival
5-15	4	20	13.41 ± 5.01^a	100	100
		40	25.31 ± 5.10^b	100	100
20-50	14	20	33.28 ± 13.08^c	100	100
		40	50.41 ± 10.58^d	100	100
60-90	24	20	38.91 ± 19.49^e	100	100
		40	89.43 ± 20.01^f	100	100

Table 8.2. The mean recovery rates, percentage recovery and percentage survival of 20-50 mm SL *Haliotis midae* after prolonged exposure to 14 g.100 ml^{-1} $MgSO_4$. Standard deviations of the means are presented. Different superscripts indicate significant differences in recovery rates at $p<0.05$.

Size (mm SL)	Conc. (g.100 ml^{-1})	Exposure time (h)	Mean recovery rate (min)	Percentage recovery	Percentage survival
20-50	14	1	74.40 ± 14.84^a	100	100
		2	103.09 ± 18.96^b	100	100
		4	171.66 ± 37.24^c	100	100
		6	246.20 ± 50.89^d	100	100
		8	365.22 ± 97.67^e	100	93.33

Prolonged exposure of the 20-50 mm SL size class to 14 g.100 ml^{-1} $MgSO_4$ revealed that the animals can be exposed for up to 6 hours to the anaesthetic without any mortalities. These exposure times are quite extreme and should theoretically never be encountered in the commercial situation, although it is encouraging to know that the animals can be exposed to $MgSO_4$ for such times before mortalities occur.

The results of Sagara & Ninomiya (1970) for $MgSO_4$ anaesthesia in *H. gigantea* confirms an earlier suggestion (Chapter 1) that the efficacy of an anaesthetic appears to be species specific. Therefore an anaesthetic which causes mortalities in one species might be very effective and not lethal in another.

In conclusion, it can be safely said that $MgSO_4$ anaesthesia would not cause any mortalities in *H. midae* in terms of commercial farming exposure times. These results also confirm earlier observations that $MgSO_4$ is indeed a very suitable anaesthetic for commercial abalone farming.

CHAPTER 9
SUMMARY AND CONCLUSION

Farming practices such as size-sorting, maintenance of proper densities, transfer between tanks, system maintenance and harvesting require the periodic removal of abalone from their holding tanks (Hahn 1989d, Tegner & Butler 1989, Shepherd *et al.* 1992, Tong *et al.* 1992). Abalone possess a large muscular foot which functions as an adhesive organ, allowing it to pull the shell down tightly onto the substratum (Fretter & Graham 1962, Barnes 1987). Moreover, dislodgement by mechanical means often results in injury and subsequent death due to lack of a blood coagulation system (Cox 1962, Armstrong *et al.* 1971, Genade *et al.* 1988, Hahn 1989d). The routine use of anaesthetics during culturing during is therefore generally and internationally accepted as being part and parcel of abalone farming.

Benzocaine is the only anaesthetic currently used in commercial abalone culture (Tong *et al.* 1992, C. Claydon, Sea Plant Products, Hermanus, pers. comm.). However, recent reports by the industry on mortalities resulting from benzocaine anaesthesia (C. Claydon, Sea Plant Products, Hermanus, pers. comm. and C. Muller, Marine Growers, Port Elizabeth, pers. comm.), suggested the need for the isolation of a safer alternative anaesthetic.

The efficacy of an anaesthetic used in aquaculture is subject to its ability to meet the requirements of the farmer. The fundamental criteria for this study was therefore to discover an anaesthetic medium and to develop a protocol that would result in mortality-free anaesthesia in *Haliotis midae*. However, restrictions on the use of anaesthetics in aquaculture relate not only to the effectiveness of the substances, but also to the legality and safety of their use (Marking & Meyer 1985). It is important to be aware of the registration status of chemicals and to avoid the use of unregistered ones. The U.S.A. Food and Drug Administration (FDA) requires that a drug used in aquaculture should be safe and effective for its intended use (Stefan 1992). This includes safety to the animal, the person administering the drug, the consumer of food products derived from the animal and safety to the environment. These were the reasons why short term detrimental effects, long term sub-lethal effects on growth, effects on muscle ultrastructure and therefore flesh texture, residues in muscle tissue and the effect of prolonged exposure were examined. Availability and cost were also taken into consideration.

Four chemicals were initially selected and their effect on isolated *H. midae* tarsal muscle was investigated. These were magnesium sulphate ($MgSO_4$), ethylenediamine tetra-acetic acid (EDTA), 2-phenoxyethanol and procaine hydrochloride. Procaine was selected because it is safe for human medicinal use and is available over-the-counter as Salusa 45 tablets (50 mg procaine hydrochloride per tablet). $MgSO_4$ was chosen because it is an unscheduled laxative and easily obtainable. It is also widely used in the food industry, and has been used as an anaesthetic in other molluscs (Kaplan 1969, Culloty & Mulcahy 1992, Heasman *et al.* 1995). EDTA was chosen because it is a calcium chelating agent and it was argued that its calcium precipitation formation would have no significant adverse effect on the consumer. 2-Phenoxyethanol was tested because it has been used widely in fish (Ross & Ross 1984, Marking & Meyer 1985, Gilderhus & Marking 1987, Yamamitsu & Itazawa 1988, Iwama *et al.* 1989, Mattson & Riple 1989, Teo *et al.* 1989, Teo & Chen 1993). All four chemicals inhibited contraction of isolated *H. midae* tarsal muscle which suggested that they all had potential as abalone anaesthetics.

Once the anaesthetic potential of the four chemicals had been established, it was necessary to develop a size-related dosage table for each of these chemicals. The criteria which had to be satisfied included mortality-free anaesthesia and an acceptable rate of anaesthesia. Consultation with the industry revealed that an acceptable anaesthesia rate for industrial purposes would be between 5 and 20 minutes (C. Claydon, Sea Plant Products, Hermanus, pers. comm.). Size-related dosage tables were also developed for two additional anaesthetics, namely benzocaine and carbon dioxide (CO_2). Only three of the six chemical, $MgSO_4$, 2-phenoxyethanol and CO_2, met the criteria of an effective abalone anaesthetic. The other three chemicals caused mortalities and were regarded as unsuitable.

The efficacy of an anaesthetic can vary widely with temperature (Gilderhus & Marking 1987) and since the temperatures at which abalone are cultured in South Africa range between 9 and 22°C the effect of temperature on the efficacy of $MgSO_4$ and CO_2 for the three size classes of *H. midae* had to be evaluated. The results clearly showed that higher concentrations of $MgSO_4$ and flow rates of CO_2 are required at lower temperatures. 2-Phenoxyethanol was not included in this evaluation, since its use resulted in mortalities during an experiment to determine the effect of regular anaesthesia on growth.

Abalone have very slow growth rates (Newman 1968, Wood 1993) and since uninterrupted growth to marketable size is an important financial consideration in a commercial farming situation, the long term sub-lethal effects of regular $MgSO_4$ and 2-phenoxyethanol anaesthesia on growth of the three size classes of *H. midae* was evaluated. The effect of CO_2 anaesthesia on growth was not evaluated as it was shown to be impractical for *H. midae* anaesthesia on a commercial scale (H.I. White & C. Claydon, unpublished data). While 2-phenoxyethanol appeared to be an effective abalone anaesthetic during the initial investigations, the increased resistance to anaesthesia and the high percent monthly mortalities noted during the growth trial led to the conclusion that it is unsuitable and unsafe for commercial application. Also, even though it is not classified as a hazardous substance, its MSDS (Material Safety Data Sheet) (Sigma Chemicals Corporation, U.S.A./Canada) states that it is harmful. This narrowed the potential list of abalone anaesthetics down to $MgSO_4$ as it was found not to affect growth and did not result in significant mortalities.

It is important that anaesthetics used during aquaculture should not affect the texture of muscle tissue, since this would in turn affect marketability. $MgSO_4$ anaesthesia did not affect the ultrastructure and by implication flesh texture of *H. midae*. The use of $MgSO_4$ should therefore not affect its marketability.

Since most anaesthetics are absorbed through the gills, residues in the tissue are likely to be found unless adequate depuration time is allowed. The FDA requires that any compound used to anaesthetize fish produced for human consumption must either be excreted or metabolized before it is consumed or the residues must be determined as being safe to the consumer (Marking & Meyer 1985). $MgSO_4$ did not leave any residues in *H. midae* muscle tissue. It is therefore a safe anaesthetic for abalone produced for human consumption.

It is also important for the abalone farmer to know how long animals can be exposed to an anaesthetic before mortalities occur. All three size classes of *H. midae* (used in this study) can be safely exposed to $MgSO_4$ anaesthesia at the required size and temperature related dosages for periods in excess of those required for routine farming procedures. In fact juvenile *H. midae* can be safely exposed to 14 g.100 ml^{-1} $MgSO_4$ for up to 6 hours.

From these investigations it can be confidently concluded that $MgSO_4$ is superior to all the

anaesthetics that have been evaluated or used for *H. midae* to date. $MgSO_4$ also fulfils the safety requirements of the FDA. It is safe for the abalone, the farmer and the consumer. It also does not affect flesh texture and leaves no residues in the muscle tissue. $MgSO_4$ is generally considered to be a non-toxic substance and is poorly absorbed by humans following oral administration (Reynolds 1982). Thus even if any magnesium were to be present in the muscle tissue by the time the product reaches the consumer, the residue levels would probably be so low that they would be completely harmless. $MgSO_4$ is an over-the-counter laxative, is generally used in the food industry and in agriculture, and is easily obtainable.

As mentioned in Chapter 4, $MgSO_4$ has also been successfully used at a commercial scale (H.I. White & C. Claydon, unpublished data). Juvenile *H. midae* (12-13 mm shell length (SL)) were effectively anaesthetized with 4 g.100 ml^{-1} BP grade $MgSO_4$ heptahydrate (epsom salts) at 14°C. All the animals recovered from anaesthesia within 20 minutes and no post-recovery mortalities were recorded. It is important for farmers to note that BP (biochemically pure) grade $MgSO_4$ should be used, since it contains less impurities than technical grade $MgSO_4$. Chemical impurities in technical grade $MgSO_4$ include chloride, calcium, iron, arsenic and lead, some of which such as Fe, As and Pb could accumulate in the flesh. Given that abalone in South Africa are farmed in baskets submerged in raceways (ten baskets per 4.5 m^3 raceway) (C.Claydon, Sea Plant Products, Hermanus, pers. comm.), the quantity of $MgSO_4$ required for anaesthesia, and therefore cost, can be significantly reduced by placing a 1 m^3 container containing the $MgSO_4$ solution on a trolley and simply placing a number of baskets in the container at one time. If necessary, the solution can be used for the rest of the baskets in that raceway.

Even though CO_2 was very effective in the laboratory, it was not effective for *H. midae* anaesthesia on a commercial scale (H.I. White & C. Claydon, unpublished data) and the costs are prohibitive.

In conclusion, the objective of this study has been met in that a suitable anaesthetic for commercial scale application for *H. midae* farming has been isolated. The data have demonstrated that *H. midae* can be effectively anaesthetized with $MgSO_4$ at regular intervals throughout the grow-out period without fear of long term sublethal effects on growth. It also does not affect the marketability of the flesh, requires no depuration period and its use does not propose any threat to the health of the consumer, the abalone farmer nor to the

environment. The data also provides the abalone farmer with adequate information with regards to dosages required for different size animals at different temperatures.

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