EFFECT OF LOW CONCENTRATIONS OF NITROUS OXIDE ON DEVELOPING RAT FOETUSES

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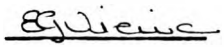
DECLARATION

I, ERICA GAYLE VIEIRA, hereby declare that this dissertation is my own work and has not been presented for any degree of another University.



The work reported in this dissertation was performed in the MRC/ University of the Witwatersrand Dental Research Institute, Johannesburg, South Africa.

To my Parents,
brother Alfred and
sister Patricia

ABSTRACT

There is an increasing concern that the pollution of operating theatre and dental surgery atmosphere with anaesthetic gases may be injurious to operating theatre personnel and dental surgery staff. A review of the relevant literature, reported in Chapter 1 of this dissertation, examined epidemiological and experimental evidence regarding this concern.

Epidemiological studies in man have provided reasonable evidence to support the hypothesis that there is an increased risk of spontaneous abortion in women exposed in the polluted atmosphere.

Experimental studies in laboratory animals, generally mice, rabbits or guinea pigs, have shown varying results depending on the anaesthetic gas used, its concentration and the length of exposure. Nitrous oxide, has been shown, however, to have teratogenic properties. Since most of the previous studies have used concentrations of nitrous oxide similar to that, which might be administered to patients undergoing general anaesthetic, a series of studies were designed to investigate the effects of low concentrations of nitrous oxide on gravid rats.

In Chapter 2 details of the environmental chambers used and the various gas mixtures and exposure times have been detailed. In the initial experimental series three groups of gravid rats were exposed to 1% nitrous oxide/air(V/V) for six hours per day, five days per week in the first, first and second and all three weeks of gestation (Chapter 3). It was found that there was statistically significant reduction in litter size, as well as body weight, body length and tail length of the offspring of the exposed rats compared to control rats. No particular pattern related to the timing of exposure was found.

In the second series of experiments gravid rats were exposed from day one to day nineteen of gestation either continuously (Chapter 4) or intermittently for six hours per day, five days per week (Chapter 5). The experimental groups were exposed to nitrous oxide/air mixtures (V/V) of 0,5%, 0,1%, 0,05%, and 0,025%, while the control groups were exposed to air only. On day nineteen the rats were killed and a detailed examination of the uterus, ovaries and foetuses was undertaken.

There was a statistically significant decrease in litter size of the dams exposed both continuously and intermittently to 0,5% nitrous oxide/air (V/V), and four of the dams exposed continuously to 0,1% nitrous oxide/air (V/V), compared to the control dams. Skeletal abnormalities were observed in 9% of the foetuses exposed continuously to 0,5% nitrous oxide/air (V/V).

The investigations reported in this dissertation have shown

(i) that the laboratory animal model system used is suitable for the examination of the effects of low concentrations of nitrous oxide on gravid rats.

(ii) that continuous 1% nitrous oxide/air (V/V) by gravid rats produced statistically significant reductions in litter size and post-natal growth of offspring.

(iii) that following continuous inhalation of 0,5%, 0,1%, 0,05% and 0,025% nitrous oxide/air (V/V), the threshold value for reproductive system defects appears to lie between 0,1% and 0,05% nitrous oxide/air (V/V).

(iv) that following the intermittent inhalation of the nitrous oxide/air (V/V) mixtures in (iii), the threshold value for reproductive system defects appears to lie between 0,5% and 0,1% (nitrous oxide/air (V/V).

The results also suggest that the 25parts/10^6 maximum pollution concentration of nitrous oxide/air (V/V) suggested by the United States, National Institute for Occupational Safety and Hygiene may be lower than necessary, although the concept that exposure of staff in polluted atmospheres should be reduced is supported. The threshold level for reproductive system defects in man remains to be determined.

PUBLICATIONS ARISING FROM THIS DISSERATION

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES

LEGENDS TO FIGURES PAGE

CHAPTER 1

INTRODUCTION

1.1 General Remarks

It has been known for many years that exposure to certain raw chemicals or industrial materials can be a serious health hazard. For instance, scrotal cancer was reported as early as 1775 to be endemic in English chimney-sweeps, liver disease is a toxic effect of chronic exposure to carbon tetrachloride in persons working in the dry-cleaning industry, cancer of the bladder has been reported in individuals chronically exposed to benzene, and spontaneous abortions occur in pregnant women chronically exposed to radiation.

Until recently, few studies searching for toxic manifestations of prolonged anaesthetic gas inhalation had been conducted. Goldman (1928) repeatedly administered nitrous oxide, ethylene and acetylene in oxygen to rats and mice for periods of up to two months and reported that no deleterious effects were observed after anaesthetisation every other day for periods averaging one hour.

Concern over occupational hazards in general anaesthesia had also been expressed by Hirsch & Kappers in 1929, who suggested that inhalation of anaesthetic agents present in the air of operating rooms might have an injurious effect on the health of surgeons and those who assist them. The matter appeared to attract little attention until 1968 when Bruce and his co-workers investigated the causes of deaths of 441 anaesthesiologists in the United States over a 20 year period. Their study suggested that anaesthesiologists have a higher than normal prevalence of both suicides and lymphoid malignancies. One year earlier, Vaisman (1967) undertook a study dealing with conditions of work. He sent a questionaire to 354 Russian anaesthetists of whom 28% used halothane, 59% nitrous oxide and 98% ether. A total of 303 replies were received of which 110 were from women. These reported that 18 of 31 pregnancies ended in spontaneous abortion.

Continuing the theme in 1973, Corbett and his associates published the results of a survey of nurse anaesthetists which indicated a much higher than expected prevalence of malignancies in the pollution group. Although all these studies relied on voluntary completion of questionaires or on data indirectly supplied by a professional society, raising the question of accuracy, the trends evident in work were similar.

Since these early studies investigations have been undertaken to determine what degree of anaesthetic pollution exists, to determine to what hazard individuals may be exposed and to determine what means may be used to lessen the hazards.

1.2 Severity of anaesthetic pollution

1.2.1 In operating theatres.

Measurement of pollution in operating theatres have included the two commonest inhalation anaesthetic agents, nitrous oxide and halothane. The concentrations of these agents have varied considerably (Table 1.1) as have the conditions in the operating theatres and the methods of specimen collection. Nitrous oxide, which is the anaesthetic gas of interest in this dissertation, ranged in concentration from a low of 70,4 parts/10^6 (V/V)(Sass-Kortsak 1981) to a high of 9 700 parts/10^6 (V/V) (Corbett 1976) in unscavenged operating theatres.

1.2.2 Pollution in Dental Surgeries

The anaesthetic gas exposure hazard also exists in dentistry probably related to the increasing use of nitrous oxide/oxygen sedation in dental surgeries. This results in the intermittent exposure of dentists and their staff to nitrous oxide pollution.

Studies by Strunin et al (1973) and Millard & Corbett (1974) of the ambient gas concentrations during dental surgery indicate that the concentrations of halothane in vented rooms may exceed 73 parts/10^6 and that the unscavenged concentration of nitrous oxide ranges from 500 to 6 000 parts/10^6.

TABLE 1.1 Nitrous oxide and halothane concentrations in scavenged and unscavenged operating theatres and Dental Surgeries.

AUTHORS	YEAR	CONCENTRATIONS IN PARTS/10^6 N_2O	HALOTHANE	COMMENTS
Linde et al	1969	428	27	unscavenged
Askrog & Harvald	1970	7000	85	inhalation zone of anaethetist
Whitcher et al	1971		3,96	mean concentration from exhalation ports
Millard & Corbett	1970	1750		after 15 minutes
		2833		after 30 minutes
		3100		after 60 minutes
Corbett	1976	330-9700	1,26	unscavenged
Swensen	1976	740,8	11,06	mean concentration 15cm in front of nasal mask
Davenport et al	1976	290,0	3,6	no precautions
		127,0	0,57	scavenged
Cleaton-Jones et al	1978	232		mean concentration in 4 surgeries
Sass-Kortsak	1981	21		6 scavenged theatres
			23	7 scavenged theatres
		70,4		4 unscavenged theatres
			1,2	3 unscavenged theatres

Cohen and his co-workers (1975) reported that waste concentrations in inhalation anaesthetic gases in the dental surgery were several times those found in hospital theatres although no figures were given.

Cleaton-Jones et al (1978) investigated the concentration of nitrous oxide at various positions in four dental surgeries in which nitrous oxide sedation was used without scavenging. They found that contamination was present in all the surgeries with the greatest concentration recorded at the dentist's nose. The highest value recorded at this site being 3643 parts/10^6.

1.3 Hazards of anaesthetic gas pollution

1.3.1 On man and animals

Hazards of anaesthetic gas pollution on man can be sub-divided into the following:

(i) blood and carcinogenicity

Lassen et al (1956) described severe bone marrow depression and resultant granulocytopaenia and thrombocytopaenia after a week of continuous exposure of tetanus patients to nitrous oxide.

Carcinogenicity of anaesthetic agents has been suggested by several reports in increased incidence of malignancy among anaesthesia personnel. Bruce and his co-workers (1968) reported a higher than expected incidence of reticuloendothelial and lymphoid malignancies among anaesthesiologists in a retrospective survey of the death certificates of 441 anaesthesiologists who died during the twenty year period from 1947-1966.

Coate et al (1979) studied the effects of prolonged exposure to low concentrations combinations of halothane and nitrous oxide on tumor incidence, especially with regard to the reticuloendothelial system. Rats were exposed for 7 hours per day, five days per week to 1 parts/10^6 and 10 parts/10^6 halothane, 50 parts/10^6 and 500 parts/10^6 nitrous oxide. No evidence of exposure-related effects on body weight, appearance, behaviour, survival or haematologic findings was found.

Histologic evaluation of the reticuloendothelial system and of other major organs revealed neither enhancement of the spontaneous tumor rate nor any unusual neoplasm. This study did not lend support to the hypothesis that these anaesthetic agents in low concentrations are responsible for the reportedly higher than average incidence of reticuloendothelial malignancies in operating room personnel.

(ii) behavioural effects

Bruce, Back and Arbit (1974) reported that inhalation of trace concentrations or enflurance Bruce and Bach (1975) significantly decreased digit recall and increased reaction time in volunteers. In 1974 Bruce et al found significant impairment at 50 parts/10^6 nitrous oxide and 1 parts/10^6 halothane. This study implied that trace anaesthetic concentrations found in operating rooms, may impair the mental function of technicians, nurses, surgeons and anaesthesiologists.

Cooke et al (1978) investigated the behavioural effects of trace and subanaesthetic halothane and nitrous oxide in man using tests of reaction time and of immediate recall, and found that sub-anaesthetic concentrations of both nitrous oxide (20-30%) and halothane (0,2%) did not impair mental function. Vessey and Nunn (1980) in their review of Occupational hazards of anaesthesia (1980) felt that it was unlikely that trace concentrations of anaesthetics have any appreciable effect on mental performance.

(iii) physiologic and psychologic effects

Chronic exposure of rats to 10 parts/10^6 halothane from conception to day 60 of their lives was found to produce permanent learning deficits, but similar exposure of adult animals was not found to cause any learning disabilities. Experiments have been performed with volunteers and of the several agents studied, halothane and enflurane appear to have significant transient effects on memory, reaction time and possible judgement. Nitrous oxide also appears to have some transient effect on these factors (Smith 1978).

(iv) reproductive system

Askrog and Harvald (1970) reported a 20% abortion rate among nurses and anaesthetists and 30% among operating room nurses. The authors stated that the results suggested a foetal lethality possibly due to anaesthetic gases, although the study did not incriminate any specific anaesthetic agent, nor was a cause and effect relationship established.

Knill-Jones et al (1972) studied the obstetric histories from 563 married women anaesthetists and 828 women doctor control subjects. The frequency of congenital abnormality, spontaneous abortion, and involantary fertility was analysed. They found that anaesthetists working during pregnancy had a significantly higher frequency of congenital abnormalities (6,5%). There was also a higher frequency of spontaneous abortion (18,2%) which was significantly different from anaesthetists not at work. Involuntary infertility was found to be twice as frequent (12%) among anaesthetists as in the control group.

Corbett et al (1974) surveyed 621 female nurse anaesthetists and found a higher incidence of birth defects among children of the exposed females. 16,4% had birth defects whilst 5,7% of children whose mothers did not work during pregnancy had birth defects.

Smith (1974) in a review stated that there are approximately 30 000 anomalous children born a year, representing 1% to 2,5% of all live births in the United States. Statistics relating cause generally ascribe 20% to genetic influence, 10% to chromosomal, and 10% to viruses, 60% are of unknown cause. It is believed that the majority of defects occuring to live births represent isolated events unrelated to specific toxic exposure. In general, it appears that toxic reactions usually manifest themselves by causing foetal death rather than malformations unless the exposure is severe enough to cause a 50% mortality. At that point, the surviving foetuses will frequently exhibit abnormalities.

It is difficult to obtain precise statistics on this subject because, like all such surveys, they suffer from the potential errors in herent in the methodology, including possible responder bias, inaccurate recall, and the possible impact of the unknown results in the non-responders, and consequently much teratologic research has been done with animals and extrapolation to man may not necessarily be correct. (Vessey and Nunn 1980). One interesting effect noted in both man and animals, is that of temporal sensitivity of exposure. According to Smith (1974) pregnant C57 mice exposed to the anaesthetic methoxyflurance (0,3%) during days 12 and 13 of the gestation period exhibit a 15% foetal death rate and a 45% incidence of skeletal malformations in the survivors, however, exposure to days 5 through 11 is without pathologic effect. Clearly, time of exposure appears to be critical factor.

Tables 1.2 to 1.6 demonstrate the spontaneous abortion rate in females exposed to anaesthetic pollution and subsequent data on malformation of their offspring.

1.3.2 On experimental animals

(i) tissues

Cohen et al (1975) studied the respiration of rat liver mitochondria in vitro during exposure to volatile anaesthetics. They found a dose-related reduction in respiration, as shown by a reduction of oxygen uptake beginning just below 1% halothane. The reduction continues until a minimum of 25% of normal is reached with halothane levels of 3% to 9%. Up to the 3% halothane level, the respiratory depression is almost totally reversible. Halothane, methoxyflurance and ether all show this reversible depression of respiration of mitochondria coincides with the inhibition of cell growth and indicates that these agents are probably lines. These results are similar to those of Jackson (1975), but his article gives more reference to cellular effects.

TABLE 1.2 Some anaesthetic health hazards occuring in exposed female personnel.

AUTHORS	YEAR	CANCER	SPONTANEOUS ABORTION IN EXPOSED FEMALES	MALFORMATIONS	STILLBIRTH	LOW BIRTH WEIGHT	INFERTILITY
Bruce et al	1968	-	-	-	-	-	-
Askrog, Harvald	1970	-	*	*	*	-	-
Cohen et al	1971	-	*	-	-	-	-
Knill Jones et al	1972	-	*	*	*	-	*
Corbett et al	1973	*	-	-	-	-	-
Rosenberg, Kirves	1973	-	*	*	-	*	-
Corbett et al	1974	-	-	*	-	-	-
Cohen	1974	*	*	*	-	-	-
Bruce et al	1974	-	-	-	-	-	-
Knill-Jones et al	1975	-	*	*	*	-	*
Pharoah et al	1977	-	*	*	*	*	-
Doll and Peto	1977	-	-	-	-	-	-
Total		2	7	7	4	2	2

TABLE 1.3 Spontaneous abortion rate in exposed females

AUTHORS	YEAR	EXPOSED SUBJECTS: WORKING AT ONSET OF PREGNANCY		EXPOSED SUBJECTS: NOT WORKING AT ONSET OF PREGNANCY		CONTROL SUBJECTS		P
		No of pregnancies	% ending in abortion	No of pregnancies	% ending in abortion	No of pregnancies	% ending in abortion	
Askrog et al	1970	229	17	85	12	-	-	NS
Cohen et al	1971	36	28	-	-	34	9	p<0,05
		337	38	-	-	58	10	p<0,01
Knill-Jones et al	1972	737	18	336	14	2150	15	p<0,05
Rosenberg & Kirves	1973	257	20	43	14	150	11	p<0,05
Cohen	1974	1826	17	676	14	1948	15	NS
		2781	20	1533	15	1948	15	p<0,001
		468	17	138	16	308	9	p<0,01
Knill-Jones et al	1975	523	16	-	-	7296	11	p<0,01
Pharoah et al	1977	670	14	-	-	8374	13	NS

TABLE 1.4 Spontaneous abortion in wives of exposed males

AUTHORS	YEAR	EXPOSED SUBJECTS				CONTROL SUBJECTS		
		WORKING AT ONSET OF PREGNANCY		NOT WORKING AT ONSET OF PREGNANCY				
		No of pregnancies	% ending in abortion	No of pregnancies	% ending in abortion	No of pregnancies	% ending in abortion	
Askrog & Harvald	1970	136	20	119	8	-	-	p<0,01
Cohen et al	1971	1350	12	-	-	54	10	NS
		237	18	-	-	54	10	p<0,05
		3416	12	-	-	1982	13	NS
Knill-Jones et al	1972	5891	11	-	-	7296	11	NS

TABLE 1.5 Malformation in offspring of exposed females

AUTHORS	YEAR	EXPOSED SUBJECTS: WORKING AT ONSET OF PREGNANCY No of infants born	EXPOSED SUBJECTS: WORKING AT ONSET OF PREGNANCY % malformed	EXPOSED SUBJECTS: NOT WORKING AT ONSET OF PREGNANCY No of infants born	EXPOSED SUBJECTS: NOT WORKING AT ONSET OF PREGNANCY % malformed	CONTROL SUBJECTS No of infants born	CONTROL SUBJECTS % malformed	P
Askrog et al	1970	191	0,5	75	0,0	-	-	NS
Knill-Jones et al	1972	893	3,0	-	-	1835	3,2	NS
Rosenberg & Kirves	1973	207	0,0	33	0,0	133	0,0	NS
Corbett et al	1974	434	8,8	261	3,8	-	-	p<0,05
Cohen	1974	1480	9,6	566	5,9	1629	7,6	p<0,05
		2210	7,7	1275	7,0	1629	7,6	NS
		384	5,9	116	3,4	276	3,0	NS
Knill-Jones et al	1975	438	1,6	-	-	6442	1,1	NS
Pharoah et al	1977	578	2,8	-	-	7317	1,8	NS

TABLE 1.6 Malformations in offspring of wives of exposed males

AUTHORS	YEAR	WORKING AT ONSET OF PREGNANCY		NOT WORKING AT ONSET OF PREGNANCY		CONTROL SUBJECTS		P
		No of infants born	% malformed	No of infants born	% malformed	No of infants born	% malformed	
Askrog & Harvald	1970	109	2,7	110	0,9	-	-	NS
Cohen et al	1971	1168	8,2	-	-	49	3,7	NS
		203	6,4	-	-	49	2,7	NS
		2988	5,4	-	-	1714	4,2	p<0,05
Knill-Jones et al	1972	5175	1,1	-	-	6442	1,1	NS

(ii) blood

Green and Eastwood (1963) found that rats exposed to 80% nitrous oxide have normal blood counts after 2 days, but significant drop in white blood counts after 4 and 15 days.

Bruce et al (1968) reported that after a prolonged exposure of rats of 0,45% halothane, a significant reduction of granulocytes in peripheral blood appears after 54 hours. No difference in lymphocyte counts in anaesthetised and in fasting control animals was found. 0,55% halothane was found to inhibit maturation of granulocytes in bone marrow.

A study was undertaken by Parbrook (1965) on the leucopaenic effects of nitrous oxide in rodents with and without surgical wounds. The animals were exposed to air, 25% nitrous oxide, 60% (rats) or 40% (mice) nitrous oxide. A leucopaenic effect and supression of the normal leucocytosis to trauma was seen with all supression of the normal leucocytosis to trauma was seen with all the concentrations of nitrous oxide after 2 days treatment. The effect of 60% nitrous oxide was greater than 25% nitrous oxide after 7 days but the difference was less pronounced in the first 4 days. The leucopaenic effect was greater in animals with surgical wounds.

(iii) reproduction system

Fink et al (1967), Sheppard and Fink (1968) demonstrated embryo-lethal and teratogenic effects following administration of 50% nitrous oxide to Sprague-Dawling rats for 24 hours per day, and on day 8 of pregnancy and continuing for 1, 2,4 and 6 days. Embryolethal and teratogenic effects increased with increasing length of exposure. The most common disturbances produced were death and resorption of embryos and abnormalities of vertebrae and ribs. The percentage of survivors and average foetal weight decreased with the duration of exposure. All experimental foetuses examined for skeletal anomalies showed defects of vertebrae ossification. Basford and Fink (1968) noted embryolethal and similar teratogenic effects in rats following administration of 0,8% halothane for 12 hours each day on day 8 - 10 of the pregnancy. Diurnal variation in the effects of halothane were noted.

Increased incidence of foetal death, congenital anomalies, and retarded growth have been reported following exposure of chick embryos to nitrous oxide, halothane, methoxyflurane, cyclopropane and diethyl ether. Ramazotto, Katz and Cupiola (1975) studied the effect of exposures to 50% nitrous oxide for 25 minutes per day at various stages of pregnancy in the rat. They found that this caused an increase in the foetal death rate.

Fifty percent nitrous oxide continuously administered to pregnant rats for 6 days will predictably increase foetal death rate as much as 57%. Subjecting chick embryos for 6 hours to high concentrations of halothane of cyclopropane alters mitotic indexes and increases embryo mortality. Similarly 36 hours of continuous exposure to 80% nitrous oxide up to 35 days showed evidence of decreased testicular weight and injury to spermatogenic cells. Animals with intermittent exposure were less affected than those with continuous exposure.

1.4 Types of chambers used in reported animal studies

Parbrook (1967) used the following method. An animal cage was placed in a clear plastic bag and the atmosphere re-circulated through soda lime and activated charcoal, water vapour condensed out in a cold trap. Fresh gases were metered into the circuit from rotameters and the oxygen concentration at 21 $\pm$ 1,5% maintained.

Wharton et al's (1978) inhalation exposures were performed in two stainless steel and plexiglass gas-tight chambers, each approximately 1 500 litres in capacity. Halothane was vapourised in a copper kettle with medical-grade compressed air and delivered through latex rubber tubing. Soda lime was placed on the floor of the chambers to absorb carbon dioxide. A high volume fan re-circulated the atmosphere within each chamber in order to maintain a uniform anaesthetic vapour concentration. Concentrations of halothane were monitored every 5 to 15 minutes using a Varian 1440 gas chromotograph and were maintained within 10 per cent of the desired concentrations.

Fink and his co-workers (1967) placed rats in a transparent box through which a gas mixture flowed at a rate of 2 litres per minutes. The mixture consisted of 45% to 50% nitrous oxide, 21% - 25% oxygen, and nitrogen for the experimental animals, and either air from a compressed air cylinder or a mixture of 21% tank oxygen and 79% nitrogen in the case of the controls. The box was provided with a wire mesh floor under which was spread a layer of soda lime carbon dioxide absorbent. The composition of the atmosphere of the box was determined twice daily by infra-red nitrous oxide and carbon dioxide and paramagnetic oxygen gas analysers.

Coate et al (1979) exposed their rats in glass and stainless steel chambers 6 cubic metres in size operated with 1,2 cubic metres/minute air flow. Rats were housed in stainless steel mesh cages with stainless steel top-loading feeders and automatic drinking valves.

1.5 Recommendations to reduce anaesthetic pollution

The National Institute for Occupational Safety and Health (NIOSH)(1977) in the United States has recommended a standard for maximum pollution with nitrous oxide of 25 parts/10^6 and 2 parts/10^6 for halogenated anaesthetics. They consider these levels to be readily achievable, but there is no evidence to suggest that they are necessarily safe levels.

1.5.1 In operating theatres

Different anaesthetic techniques and various circuits nearly always produce excess gas, which usually enters the room. Several techniques to prevent this have been developed.

According to Berner (1978) and NIOSH (1977) a scavenging system should consist of three major components:

- *a collecting device to lead waste anaesthetic gases to*
- *a disposal route to carry pollutive gases from the operating rooms and*
- *a method or device for limiting positive and negative pressure variations in the breathing circuit, which may be caused by ventilation and the scavenger equipment.*

Open scavenging systems, when accurately dimentioned, seem to offer a trouble-free and safe alternative to simple evacuation of anaesthetic waste gas (Paloheima et al 1979).

Although properly functioning scavenging systems are important to a pollution-free atmosphere, leak-free ventilators and other anaesthetic equipment are also essential and these should be tested regularly (NIOSH 1977). Improper anaesthetic practice in the form of intentional outflow or discharge from the anaesthetic breathing circuit during different stages of anaesthetic is also an important source of pollutive gases. (Millard and Corbett, 1974 and Cleaton-Jones et al 1978).

1.5.2 In dental surgeries

As in the case of operating theatres anaesthetic gas removal from dental surgeries is also important (Millard et al 1974, Parbrook and Davis 1979), the most common gas in this case being nitrous oxide.

The health hazard of nitrous oxide mentioned earlier apply also to the exposed dentist and staff strongly suggesting that preventative measures should be taken. However, the provision of protection in the dental surgery from the hazard of anaesthetic exposure poses a difficult one. Unlike the hospital operating rooms, most air-conditioning systems used in dental surgeries are of the re-circulating type that provide continuing recirculation of the waste anaesthetic gases. In addition high flow of anaesthetic gases used, in combination with the open mouth of the patient makes the application of scavenging techniques difficult.

At present methods of using waste gas scavenging within dental surgeries are currently under investigation. These include the use of individual, fresh air, breathing masks, the use of controlled air flow pattern that rapidly evacuates the air immediately adjacent to the patient and clincian, and the use of an exhaust line attached to the evacuation part of the breathing circuit.

In dental surgeries, as in operating theatres, precautions to reduce anaesthetic gas pollution must be taken. This point has been emphasised in general recommendations made by the National Health and Medical Research Council into the hazards of exposure to anaesthetic agents (1977), are based on the evidence presented earlier in this chapter and indicate current thinking on anaesthetic gas pollution.

(a) It is ethically, scientifically and economically justifiable to install in operating rooms, scavenging equipment of proper construction and reasonably uniform design as soon as possible.

(b) A monitoring programme, appropriate to the case and size of the institution should also be adopted.

(c) Pregnant women should be informed that there may be a hazard to their pregnancy in the operating theatre environment, and be entitled to opt for alternative employment. Dental surgeries in which anaesthetic gases and vapours are regularly used should also be scavenged.

1.6 Aims of the study

From the review of the literature it is clear that steps must be taken to minimise anaesthetic gas pollution. It is also clear that no threshold exposure level for health hazards exist. The studies reported in this dissertation were undertaken therefore to:

1. Develop a laboratory model system to produce defects of the reproductive system in laboratory animals through inhalation of low concentrations of nitrous oxide.
2. To investigate threshold concentrations of nitrous oxide that would produce such reproductive defects.

Nitrous oxide was chosen as the anaesthetic gas for investigation since it it the commonest anaesthetic gas used in the operating theatre and dental surgery.

CHAPTER 2

MATERIALS AND METHODS

This chapter deals with general methods and materials used in the investigation. Where appropriate specific methods will be described in subsequent chapters. The experimentation reported in the thesis was approved by the Animal Ethics Committee of the University of the Witwatersrand, Johannesburg, prior to commencement of the investigations.

2.1 Experimental Animal

The experimental animal chosen was the Wistar Albino rat as this animal has proved to be a good experimental model for studies on nitrous oxide pollution (Vieira et al 1978). It is also the strain of laboratory rat that has been used in the Dental Research Institute for more than 25 years, thereby ensuring familiarity with the animal. A further reason for its selection is that Fink et al (1967) have reported that there is a similarity in the susceptibility to nitrous oxide in rats and man, and they have suggested that studies on the effects of nitrous oxide in the rat indicate possible effects of nitrous oxide in man.

2.2 Environmental Chamber

The environmental chamber used was that designed and constructed by Austin, Vieira and Cleaton-Jones (1979) (Figure 2.1). This has been described in detail in that publication but may be summarised as follows:

Two chambers each hold four polypropylene rat cages in a vertical tier, each cage having a lateral wire mesh opening to aid gas entry into the cage (Figure 2.2).

Gases are introduced along one side of the chamber from accurate flow meters via a centrifugal fan to ensure gaseous mixing and are extracted from the opposite side by a further centrifugal fan.

The flow rates of gases into the chamber, carbon dioxide, ambient temperature, relative humidity, water vapour and ammonia have previously been investigated (Austin et al 1979).

Similarly the nutritional status of rats housed in the chamber has been shown by Austin et al (1979) to be unaffected.

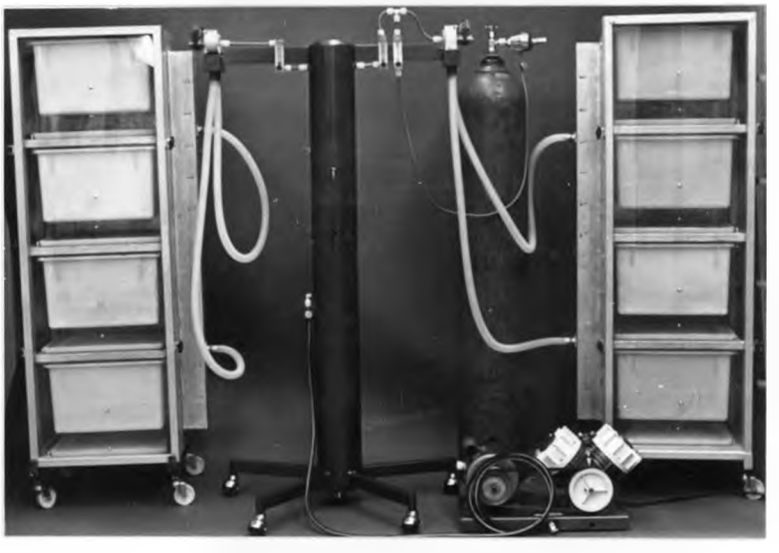


Figure 2.1 Inhalation chambers with central gas metering and mixing apparatus.

Figure 2.2 Open chamber showing air supply and extraction system and modified rat cage. Note the wire mesh bottom and wire mesh grid on the cage side.

2.3 Housing of the rats

Three female rats were housed in each cage in an environmental chamber for a period of 7 days before commencing the investigations. During this period, there were no male rats in the chambers and only compressed air was supplied to the control and experimental chambers.

2.4 Feeding

The rats were fed on a standard laboratory ration, Epol Rat Cubes, (Vereeniging Consolidated Mills), and food and water were available ad libitum to all rats during the aclimatization and experimental periods.

2.5 Gaseous flow rates

The experiments were carried out at an altitude of 1700m with a mean barometric pressure of 82,6kPa. At the commencement of each experiment nitrous oxide was added to the air supply of one chamber by means of a calibrated flow meter to provide the required concentrations of nitrous oxide in air (V/V) (Figure 2.3). These were 1% (0,83kPa), 0,5% (0,41kPa), 0,1% (0,08kPa), 0,05% (0,04kPa), 0,025% (0,02kPa). The gases were thoroughly mixed by passage through a centrifugal fan before delivery to the chamber.

Earlier experimentation (Austin et al 1979) had shown that at a flow rate of 35 litres/minute (1 air change/7 minutes) a complete turnover of gas contents occured only every 14 minutes (Figure 2,4). On this basis it had been calculated the concentrations of nitrous oxide were restored to their original values 15 minutes after the chambers were restored to operation after the daily 10 minute period when the chambers were opened for excreta removal and food replenishment.

2.6 Monitoring of chamber environment

During the experimental period nitrous oxide, carbon dioxide and humidity were monitored by gas sampling (Figure 2.5) and the concentrations detected with a katharometer in a Pye Unicam Gas Chromatograph calibrated as follows:

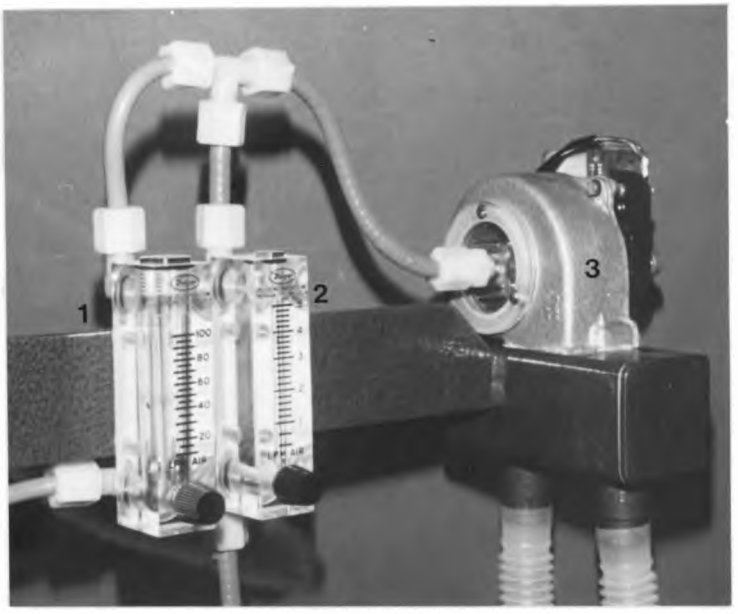
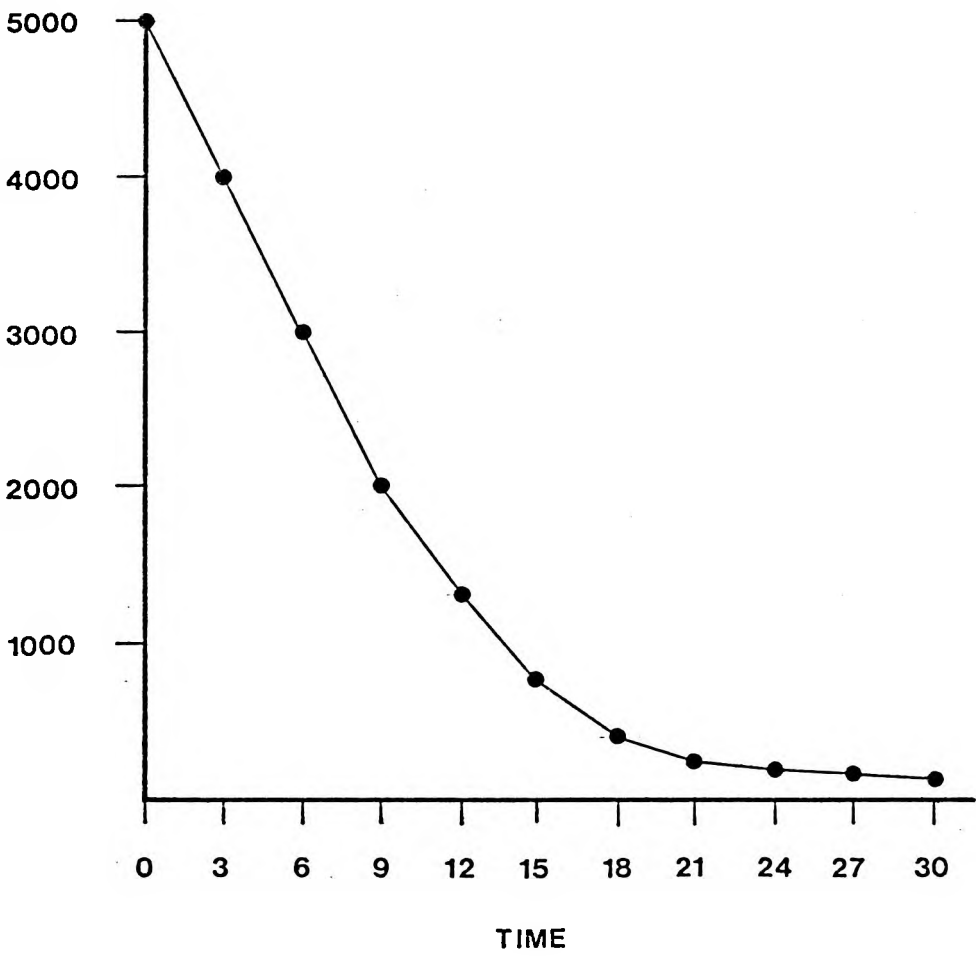


Figure 2.3 This picture shows the flow meters. On the left is the flow meter (1) for the compressed air and on the right the flow meter (2) for the control of the nitrous oxide flow rate. On the far right is the centrifugal fan used for mixing the gases (3).

FIGURE 2.4 Ventilation rate as shown by tracer gas dilution method.



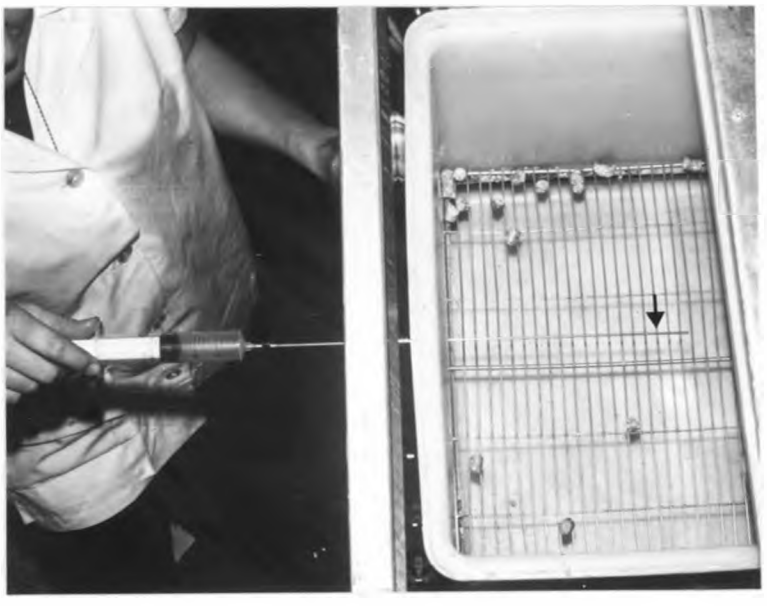


Figure 2.5 Sampling of gas inside one of the cages in the environmental chamber. The needle used to draw up the gas sample is arrowed.

Column	2m x 0,6350cms o.d. glass packed with Porapack: Q 80/100 mesh
Column temperature	80°C
Injector temperature	150°C
Detector	Thermal conductivity detector
Detector oven	100°C
Detector filament	150°C
Carrier gas	Helium at 30ml/minute
Measurement of peaks	Peak areas : Pye Unicam DP88 computing integrator

Although no concentration gradients were found within the chambers during their development and construction the positions of the cages were changed daily to randomise any positional effects.

2.7 Mating of the rats

The female rats were mated in the evenings by placing three female rats with one male rat at 16h00 into a cage in the environmental chambers into which only air was delivered. The following morning at 06h00 the male rats were removed, copulation plugs were looked for an vaginal smears made in the following manner. Each animal was loosely held while a match was then inserted into the vagina (Figure 2.6). The match was then removed and the match head rotated into a drop of distilled water on a glass slide (Figure 2.7) over which a coverslip was placed. The smears were then examined under the light microscope and pregnancy diagnosed if sperm were seen (Figure 2.8, 2.9). The time of conception was considered to be midnight of the previous evening. Thus, the day of the smear was designated day zero, the next day one, and so on (Nanda, 1969).

2.8 Isolation of gravid rats

Each pregnant rat was isolated shortly before giving birth. At this stage, the rats were placed in a special room where there was an absence of noise and the author was the only person to handle them, to feed them, as well as to clean the cages. The reason for this was to ensure standardization of births. Once born, the offspring were counted and the number recorded to be the number of young rats in the litter.

Figure 2.6 A match is gently inserted into the vagina of the rat, to pick up vaginal contents on the match-head.

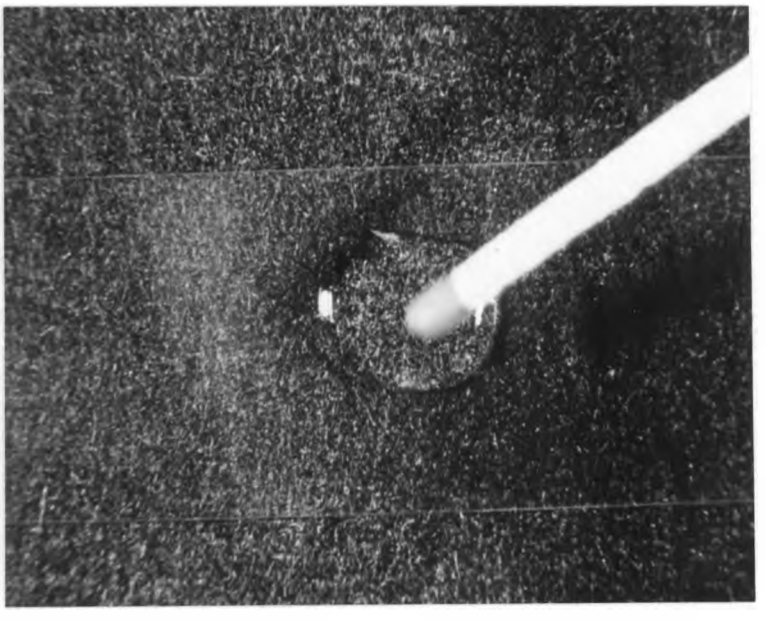


Figure 2.7 The match head is rotated in a drop of distilled water on a glass slide.

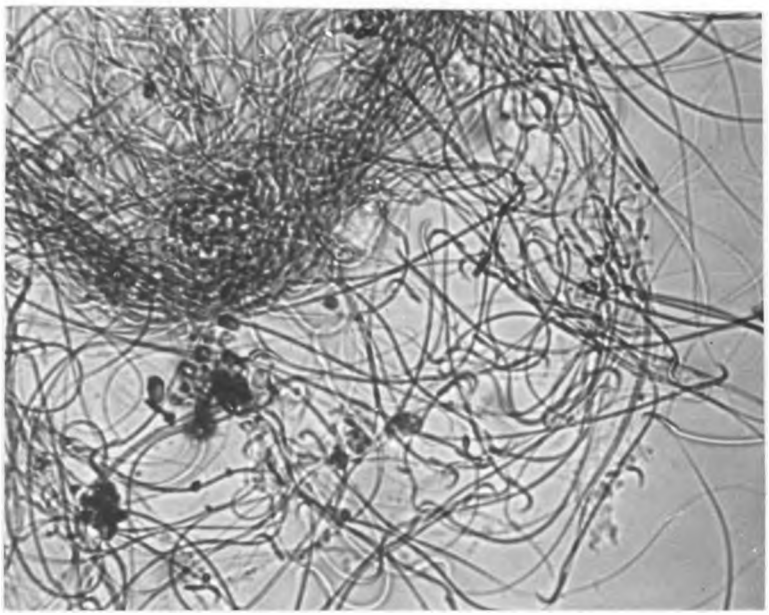


Figure 2.8 A positive vaginal smear showing the presence of spermatozoa.

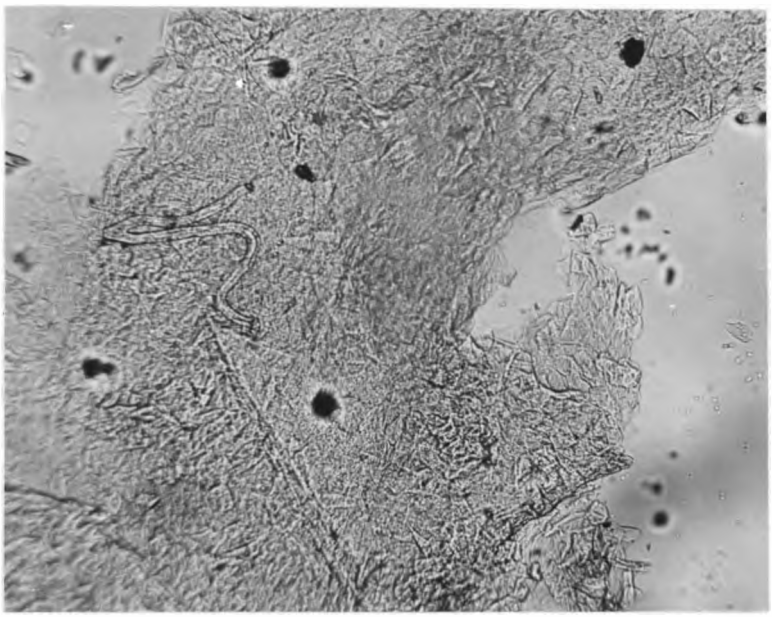


Figure 2.9 A negative vaginal smear showing the presence of epithelial cells.

2.9 Weighing of offspring

At weekly intervals for 8 weeks postpartum the offspring were weighed. For the first three weeks a chemical balance was used (Figure 2.10) and thereafter a kitchen scale. All measurements were to the nearest 0,1 gr. (Figure 2.11).

2.10 Measuring of offspring

After birth, at weekly intervals the tail and body lengths were measured. The measuring apparatus consisted of a board graduated in millimetres, with a vertical slotted metal plate attached to it (Figure 2.12) (Jeffreys, 1969). Each rat's tail was placed in the slot and was drawn through until the haunches were against the plate. The distance to tail tip was read off the scale in millimetres, to the nearest 0,5 of a millimetre. After flattening the body of the rat against the scale the distance was read to the tip of the nose (Figure 2.13).

2.11 Sexing of the Offspring

The rats were sexed by external characters using a binocular operating microscope. The male is characterised by a larger papilla and a greater distance between the anus and genital papilla (Figure 2.14) (Farris and Griffith, 1971).

2.12 Examination of foetuses

When the experiment was terminated just before birth the gravid rats were killed by carbon dioxide administration, the foetuses were removed from the uteri and each foetus was systematically examined for any external malformations using a dissecting microscope (Figure 2.15). The body cavities were opened by a mid-ventral incision and a systematic examination of the internal organs was then undertaken (Figure 2.16), with the dissecting microscope.

Each foetus was numbered and placed in a seperate specimen bottle. The foetuses were fixed in 10% buffered formal saline (di-sodium hydrogen orthophosphate, sodium di-hydrogen orthophosphate, sodium chloride). for a period of 7 days. Thereafter the foetuses were cleared in 0,5% potassium hydroxide after which the foetal skeletons were stained with alizarin red.

Figure 2.10 Weighing a one week rat on a chemical balance.

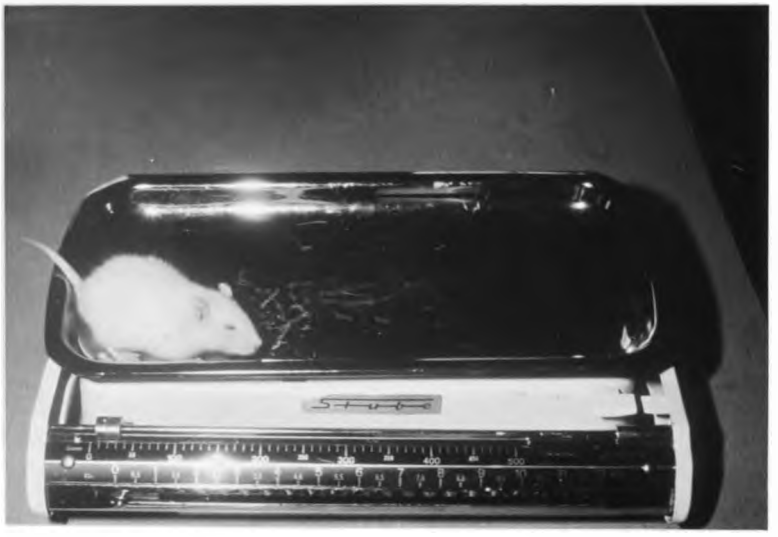


Figure 2.11 Weighing a young rat on a kitchen scale.

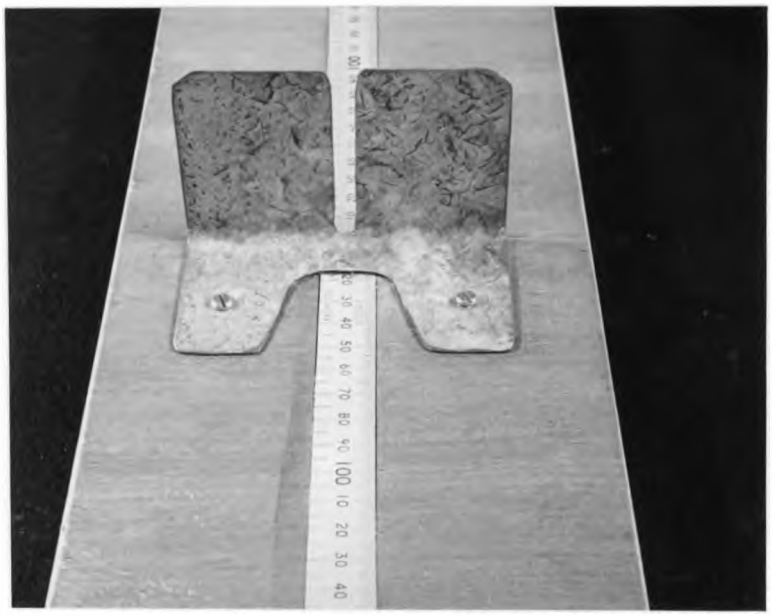
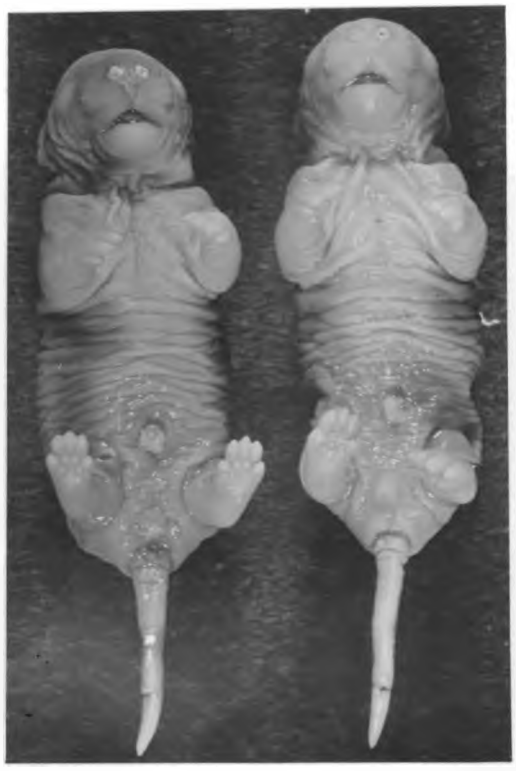


Figure 2.12 Measuring apparatus used for body length and tail length measurements. It consists of a graduated board having graduations in millimetres, with a vertical slotted metal plate attached to it.

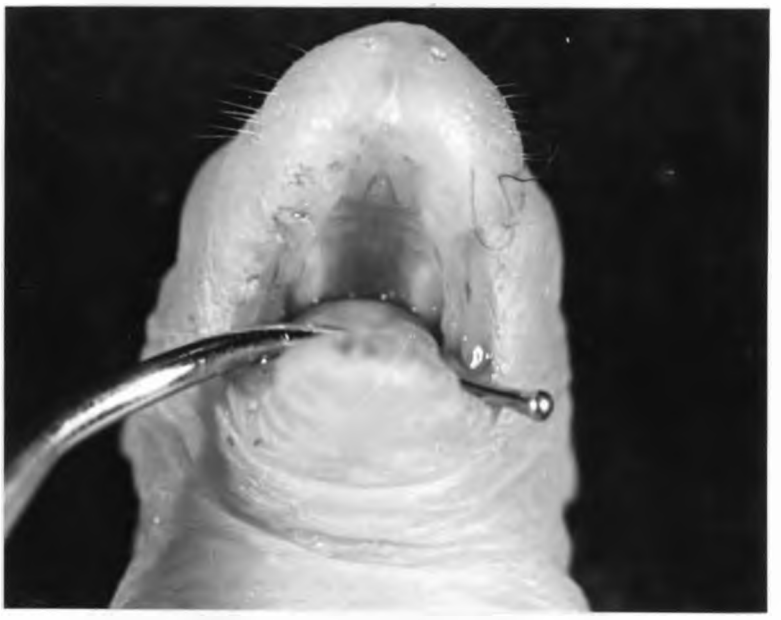
X2.2

Figure 2.13 Young rat being measured. Rat's tail is placed in the metal slot and is drawn through till the haunches are against the plate. After flattening the body of the rat against the scale the distance is measured to the tip of the nose and tail. The tail was straightened before the measurement was taken.



X2.2

Figure 2.14 The male rat on the right is characterised by a larger genital papilla and a greater distance between the anus and genital papilla than the female on the left.



X5.9

Figure 2.15 Intact palate seen during external examination of foetus using a dissecting microscope.

X3.4

Figure 2.16 Internal examination of foetus using a dissecting microscope. Note the large foetal liver.

A systematic examination of the foetal skeletons was carried out in random order by placing the foetuses on a light box and any anatomical variations being recorded and photographed. The randomization was carried out as follows: as the foetuses were already numbered these numbers were placed in a container, shuffled and then the numbers drawn, thus determining the order of the foetal examination. When anatomical variations were found these were compared with litter mates and litters belonging to the control group.

2.13 Crown-rump measurements

Crown-rump measurements were made using a modified version of the method described by van Rensburg (1976). A steel pin was cemented to the floor of a petri dish. To this pin a short length of 4/0 multi filiament stainless steel suture wire was fastened at one end to provide a measuring device. The alizarin stained foetuses of the control and nitrous oxide exposed groups were positioned so that the coronal suture line was opposite and in contact with the pin. The suture wire was drawn along the midventral dorsal surface of the foetuses and clamped with artery forceps at a point adjacent to the fourth coccygeal vertebrae. The wire was then straightened and the distance between the pin and artery forceps measured with vernier calipers to the nearest 0,1mm (Figure 2.17). This distance was taken as the crown-rump length of the foetuses.

2.14 Statistical analysis

The experimental format was a two-way nested design. The statistical analysis consisted of a two-way analysis of variance based on the design. All statistical analysis was carried out using a Hewlett Packard HP 33E calculator, and the following formulae:

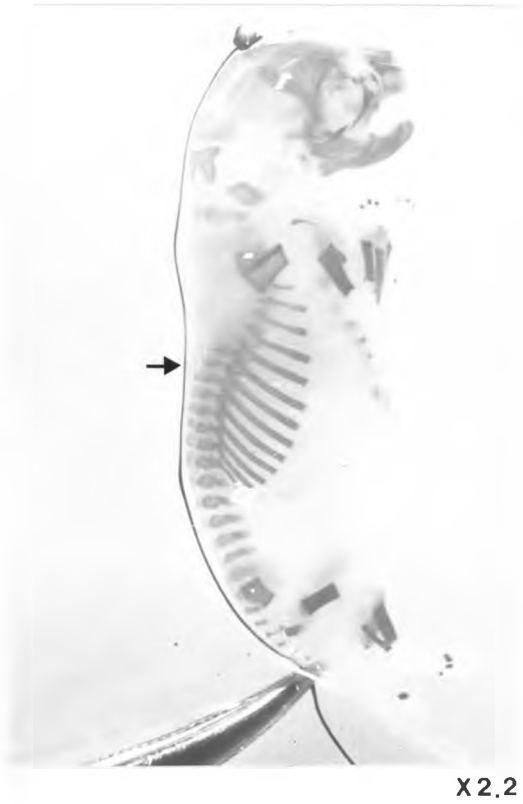


Figure 2.17 Crown-rump measurement of a foetus. The suture wire (arrowed) was drawn along the mid-ventral dorsal surface of the foetus and clamped with artery forceps at a point adjacent to the fourth coccygeal vertebrae.

TWO WAY FORMULAE USED IN ANALYSIS OF VARIANCE

Source of Variation	Sums of Squares	Degrees of Freedom	Mean Squares
Between main groups	$\Sigma N_{i\cdot} \bar{x}_{i\cdot\cdot}^2 - N_{\cdot\cdot} \bar{x}_{\cdot\cdot\cdot}^2$	2 - 1 = 1	S_1^2
Between sub-groups (Within main group)	$\Sigma\Sigma N_{ij} \bar{x}_{ij\cdot}^2 - \Sigma N_{i}^2{}_{\cdot\cdot}$	$N_{i\cdot} - 2 = 22$	S_2^2
Residual (Within sub-groups)	$\Sigma\Sigma\Sigma x_{ijk}^2 - \Sigma\Sigma N_{ij} \bar{x}_{ij}^2{}_{\cdot}$	$N_{\cdot\cdot} - N_{i\cdot}$ = $N_{\cdot\cdot} - 24$	S_3^2

The table was divided into four groups:

1. Source of variation - between main groups
   between sub-groups
   Residual

2. Sums of variation

   where x_{ijk} is the observation on the k^{th} foetus in the j^{th} litter in the i^{th} group.

   N_{ij} = number of individuals in j^{th} litter in i^{th} group

   $N_{i\cdot}$ = number of individuals in i^{th} group

   $N_{\cdot\cdot}$ = total number of all animals

   $\bar{x}_{ij\cdot}$ = mean of j^{th} litter in i^{th} group

   $\bar{x}_{i\cdot\cdot}$ = mean of all litters in i^{th} group

   = sum

3. Degrees of freedom

4. Mean squares which are S_1^2, S_2^2, S_3^2

   $\bar{x}_{\cdot\cdot\cdot}$ = mean of all litters in all groups

Final calculation

From the data obtained from the 1. sums of squares
2. degrees of freedom
3. mean values

the following were computed.

a) statistic for difference between main groups $F_1=S^2_1/S^2_3$

b) test statistic for difference between sub-groups $F_2=S^2_2/S^2_3$

The probability values of the calculations were finally obtained from standard F value tables.

In the case of litter size, foetal resorption and skeletal malformations, the Student's 't' test for two independent groups, Chi-square test and Fisher's exact probility test were used, respectively (Siegel 1956).

2.15 Inter-and intra-examiner reproducibility

There were two examiners, the author and a final year veterinary student, who carried out all the measurements and calculations independently. Thirty percent of the measurements were re-done and Student's 't' test for paired samples applied to test for statistically significant inter-and intra-examiner variation. No significant variations were found.

CHAPTER 3

INTERMITTENT EXPOSURE OF GRAVID RATS TO 1% NITROUS OXIDE/AIR(V/V) AND THE EFFECT ON THE POSTNATAL GROWTH OF THEIR OFFSPRING

3.1 Introduction

In Chapter 1, it was shown how the conditions of experimental exposure to mixtures of nitrous oxide and air, reported in the literature vary considerably. No standard method exists. The work reported in this Chapter was undertaken as a pilot investigation to determine whether the model system used would produce reproductive system defects.

In this study, 1% nitrous oxide/air(V/V) was chosen as the experimental concentration because this approximates the highest reported nitrous oxide pollution level recorded in a dental surgery by Millard and Corbet (1974) at the time that the investigation was begun. The exposure time of 6 hours a day, 5 days per week was selected to simulate nitrous oxide levels which might be encountered during a dentist's working day when nitrous oxide sedation is used.

3.2 Materials and Methods

Forty-eight Wistar Albino rats were obtained from the South African Institute for Medical Research and divided into six groups of eight rats each - three experimental groups and three control groups. In the first experimental group eight rats were exposed to nitrous oxide for their entire gestation. The second group was exposed for the second and third weeks of gestation and the third group for the first week only. Random allocation to the groups was carried out as follows: each rat was numbered and the numbers were placed in a container. In a seperate container were numbers pertaining to the types of exposure. The containers were shuffled and cards drawn from each container giving details of rat number and experimental exposure period.

The rats were placed in the environmental chambers in modified cages as discussed in Chapter 2, and housed in the environmental chambers for a period of 7 days prior to commencement of the studies to enable them to adjust to their new environment. During this period only compressed air was supplied to the control and experimental chambers. At the onset of the experiments nitrous oxide was added to the air supply of the one chamber by means of a calibrated flow meter as discussed in Chapter 2.

At the conclusion of the exposure times the gravid rats were isolated shortly before giving birth. Once born, the offspring were counted and the number recorded as the actual number of offspring in the litter. Measurements of the offspring were carried out at weekly intervals for 8 weeks as described in Chapter 2.

3.3 Results

3.3.1 Litter size

A total of 410 rats were born of which 248 were control rats and 162 were experimental rats. The latter group comprised 66 from mothers exposed for all three weeks, 50 from mothers exposed for the first two weeks and 46 from mothers exposed only for the first week.

The mean number of young rats per litter are shown in Table 3.1. In the control group there was a total of 248 with a mean number of 10,3 offspring per litter. In the groups exposed to nitrous oxide for 3 weeks, first and second weeks and first week only the total number of offspring was 162 with 66, 50 and 46 offspring per group, respectively, and with a mean of 8,3 in the group exposed for 3 weeks, 6,3 in the group exposed for the first and second weeks, 5,7 in the group exposed for the first week only. There were significantly less rats per litter in the mothers exposed to 1% nitrous oxide/air(V/V) for the first two trimesters ($P<0,001$) and for only the first trimester ($P<0,001$).

The mean litter size of the mothers exposed to the gas throughout their pregnancy also differed significantly from that in the control group.

TABLE 3.1

LITTER SIZE FOR VARIOUS RAT GROUPS

	Period of exposure to 1% N_2O/Air	Number of Litters	Total offspring	Rats per litter $\bar{x}$ ± SD	Student's 't' test (Control vs test group) t	P value
Control group	none	8	248	10,35 ± 2,3	-	-
1	3 weeks	8	66	8,3 ± 3,2	6,4	P<0,001
2	1st & 2nd weeks	8	50	6,3 ± 2,6	11,2	P<0,001
3	1st week	8	46	5,7 ± 1,6	12,4	P<0,001

3.3.2 Body weight

In Table 3.2 the mean body weight of both the experimental and control animals are listed as well as the values obtained through pooling of all the results on all the experimental animals. Trends for each group are shown in Figures 3.1 - 3.4. There was a difference in body weight between the 3 groups for the 8 week period. All the experimental and control groups differed significantly for main and sub-group differences except at 7 weeks. At this point, only the main group effect was significantly different from the control group ($P<0,05$). It was found that the P values for differences between the main groups and sub-groups were the same except for seven weeks where the values differed. No differences in sub-group effect was detected.

3.3.3 Tail length

Table 3.3 shows the results obtained in the tail length measurements. Trends for each exposure period are shown in Figures 3.5 - 3.8. At one week there was no statistically significant difference between the tail lengths for the two groups of young rats, but thereafter there were significant differences betwen the control and experimental rats. The tail length measurements of the offspring exposed to 1% nitrous oxide/air(V/V) for their entire gestation and those exposed for the first and second weeks of gestation appeared to have similar measurements throughout the eight weeks postpartum period. The offspring exposed for the first week of gestation had shorter tail lengths than the groups exposed prenatally for three weeks and for the first two weeks to the 1% nitrous oxide/air(V/V) concentration. This was notable at weeks one, two, four, five seven and eight weeks.

3.3.4 Body length

The growth in body length followed a similar pattern to that of the growth rate of the tail (Table 3.4). Once again trends for each exposure period are shown in figures 3.9 - 3.12.

TABLE 3.2 Body weight in grams of offspring

Weeks post partum	Control (n = 248)			Period exposed to N_2O								
				Pooled values for all N_2O-exposed groups			Three weeks (n = 66)			First and second weeks (n = 50)			First week (n = 46		
	$\bar{x}$	±	SD	$\bar{x}$	±	SD	$\bar{x}$	±	SD	$\bar{x}$	±	SD	$\bar{x}$	±	SD
1	10,0	±	1,2	7,0	±	1,2 **	8,1	±	1,6	8,3	±	1,4	6,5	±	0,53
2	17,6	±	4,7	15,5	±	4,83**	17,2	±	2,7	16,1	±	4,0	13,4	±	7,8
3	31,6	±	4,5	24,5	±	6,13**	27,7	±	2,8	24,8	±	4,8	20,9	±	10,8
4	42,3	±	3,9	39,1	±	4,27*	39,4	±	3,5	41,9	±	5,6	31,2	±	13,7
5	54,0	±	10,3	52,8	±	10,27**	50,6	±	5,6	58,0	±	14,3	50,0	±	10,9
6	67,0	±	7,7	65,0	±	11,23**	64,7	±	8,7	68,4	±	10,7	62,3	±	14,3
7	85,6	±	9,3	80,2	±	14,20*	81,5	±	17,7	85,0	±	18,3	74,2	±	6,6
8	107,8	±	8,10	90,7	±	15,80**	91,3	±	21,0	94,3	±	21,2	86,7	±	5,2

P values for main group effects * P<0,05, ** P<0,001

FIGURE 3.1 Graph showing mean body weight by age in weeks postpartum for offspring of rats exposed for all three weeks. One standard deviation is indicated.

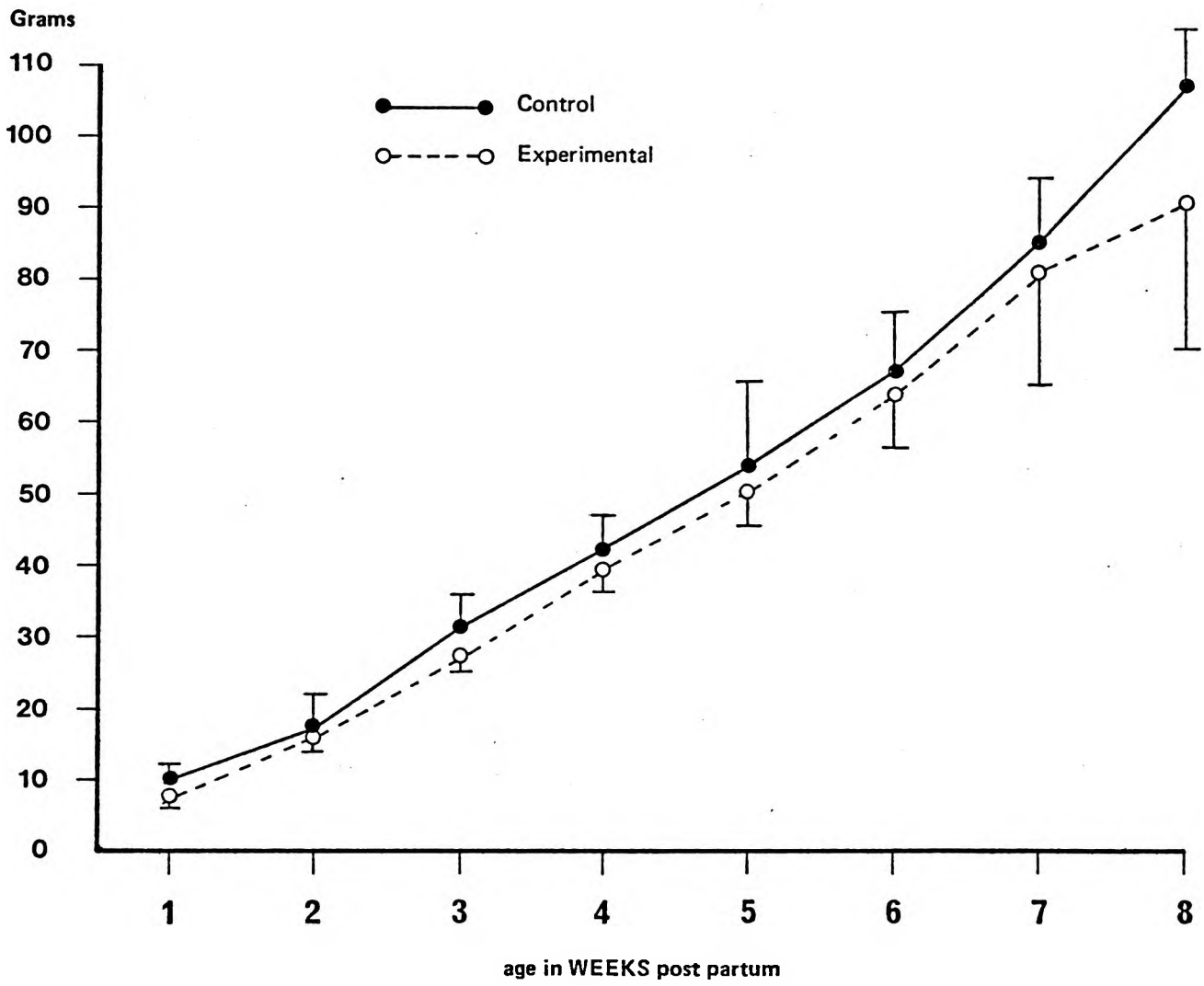


FIGURE 3.2 Graph showing mean body weight by age in weeks postpartum for offspring of rats exposed for first and second weeks. One standard deviation is indicated.

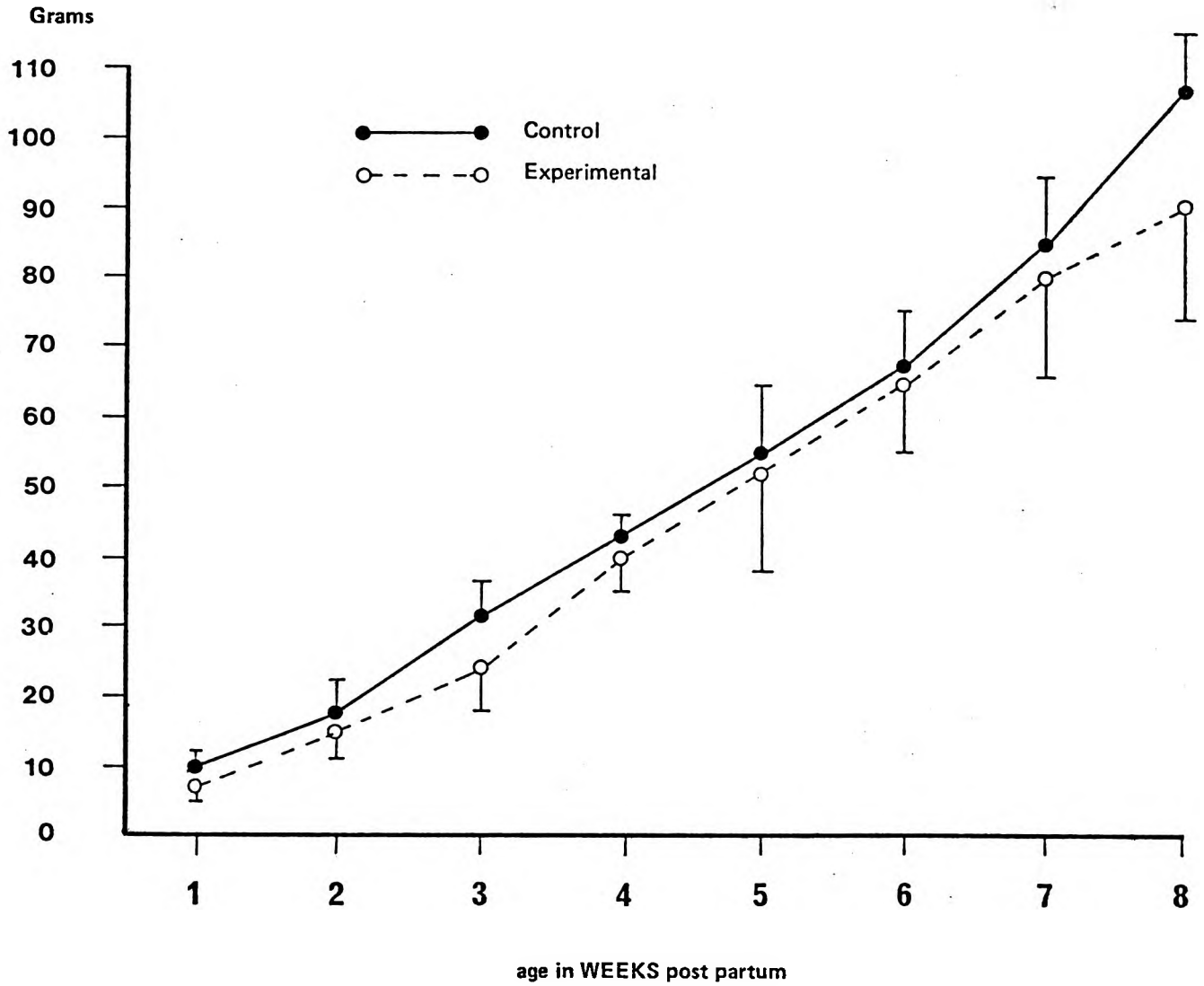


FIGURE 3.3 Graph showing mean body weight by age in weeks postpartum for offspring of rats exposed for first week only. One standard deviation is indicated.

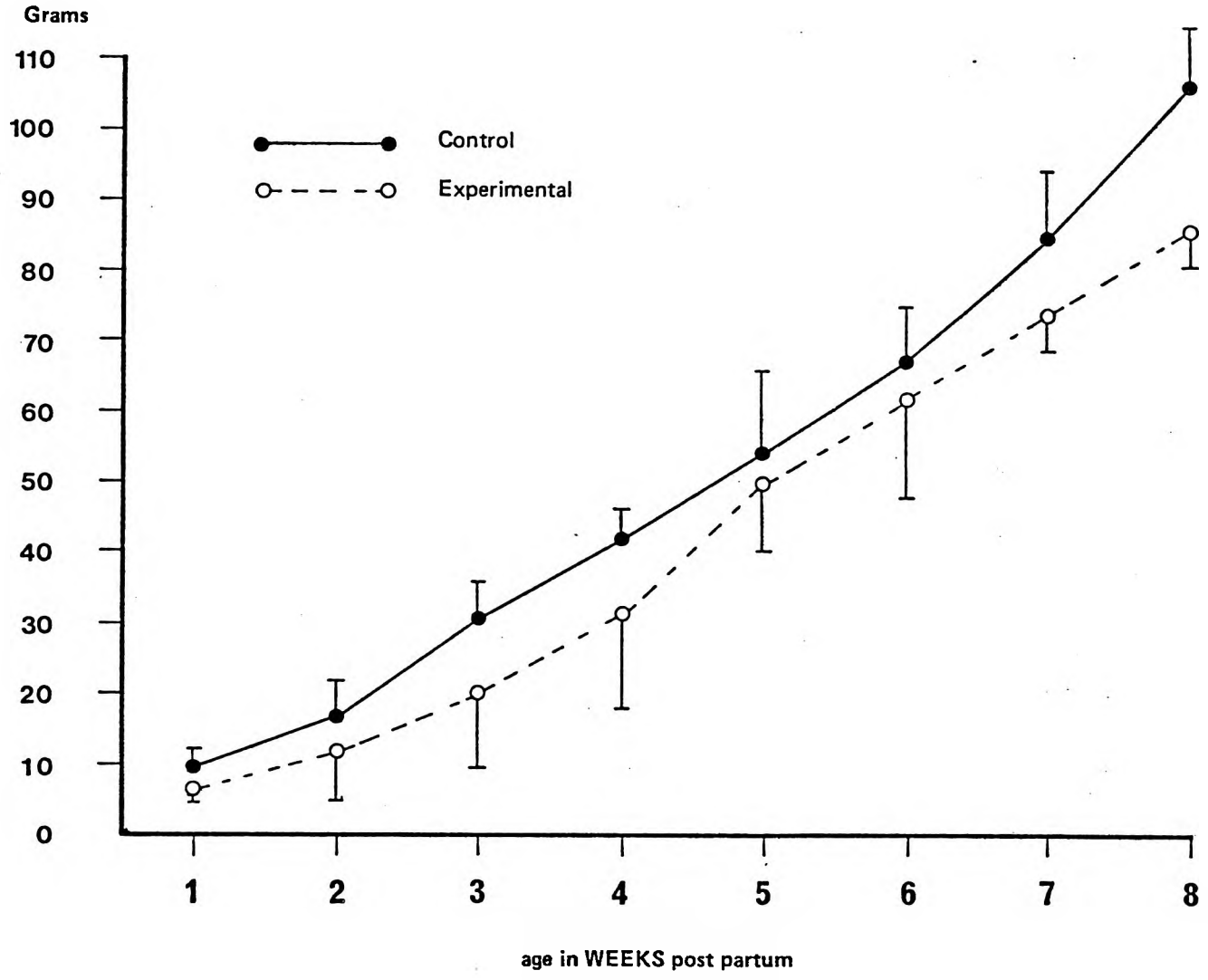


FIGURE 3.4 Graph showing mean body weight by age in weeks postpartum for offspring of rats (pooled results). One standard deviation is indicated.

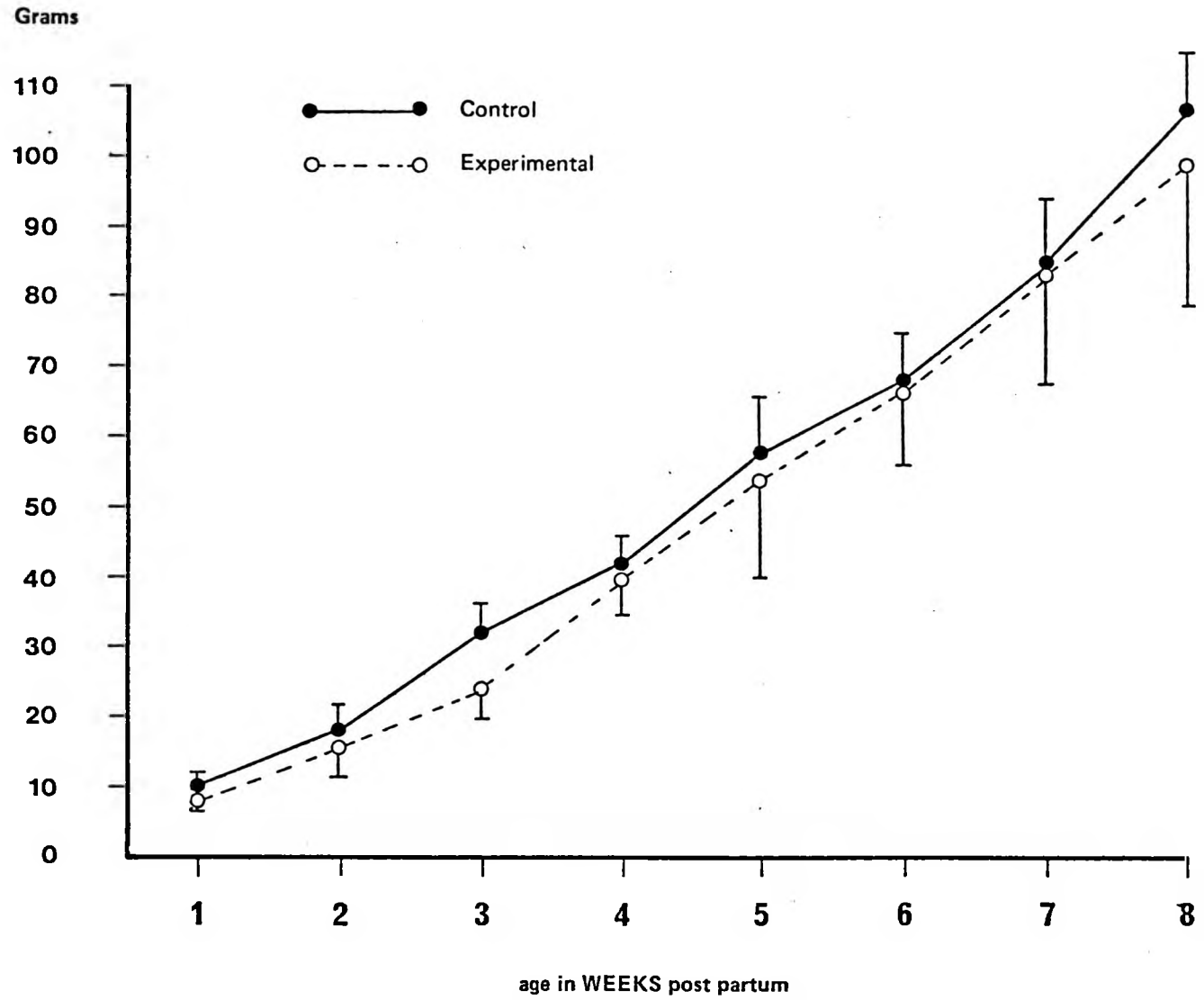


TABLE 3.3 Tail length in millimeters of offspring

Weeks post partum	Control			Period exposed to N_2O								
				Pooled values for all N_2O-exposed groups			Three weeks			First and second weeks			First week		
	$\bar{x}$	±	SD	$\bar{x}$	±	SD	$\bar{x}$	±	SD	$\bar{x}$	±	SD	$\bar{x}$	±	SD
1	54,0	±	4,1	23,0	±	4,6	24,6	±	4,0	25,4	±	5,8	20,6	±	4,1
2	71,0	±	3,7	36,2	±	4,6	38,6	±	2,2	38,6	±	6,3	28,5	±	5,3
3	78,1	±	5,9	43,0	±	6,6**	48,7	±	5,8	42,8	±	6,4	40,1	±	7,7
4	95,1	±	14,2	62,0	±	6,4**	66,9	±	11,8	68,6	±	3,5	50,5	±	3,9
5	118,7	±	7,0	80,0	±	10,3**	82,3	±	13,8	88,1	±	5,3	67,1	±	12,8
6	132,4	±	8,8	89,1	±	5,8**	80,8	±	2,5	95,6	±	13,1	90,8	±	2,0
7	147,7	±	9,8	103,9	±	7,9*	105,6	±	10,5	108,6	±	10,7	97,5	±	2,7
8	159,2	±	6,9	110,9	±	10,63**	111,9	±	12,8	115,0	±	12,5	105,8	±	6,6

P values for main group effects * $P<0,02$, ** $P<0,001$

FIGURE 3.5 Graph showing mean tail length by age in weeks postpartum for offspring of rats exposed for all three weeks. One standard deviation is indicated.

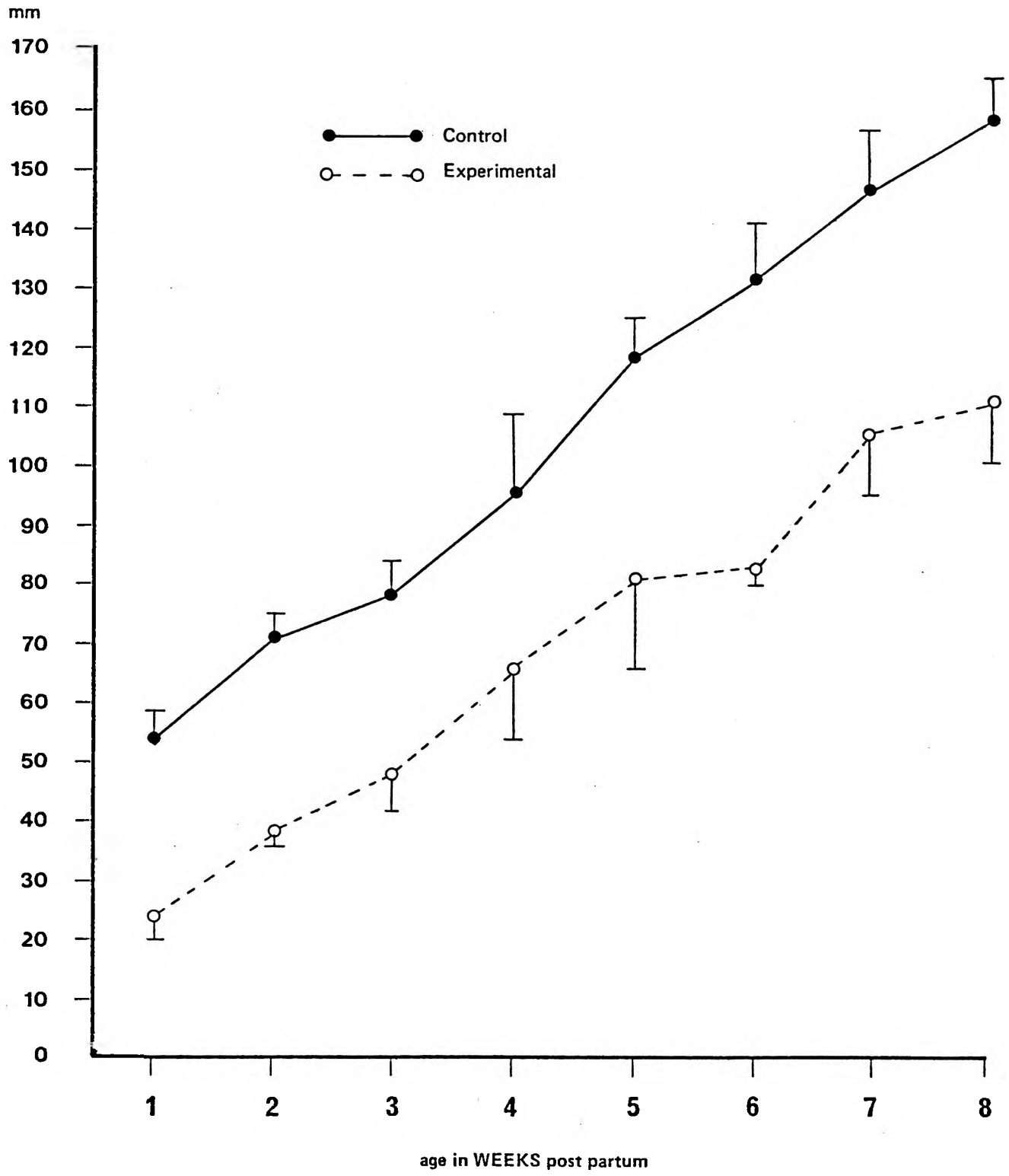


FIGURE 3.6 Graph showing mean tail length by age in weeks postpartum for offspring of rats exposed for first and second weeks. One standard deviation is indicated.

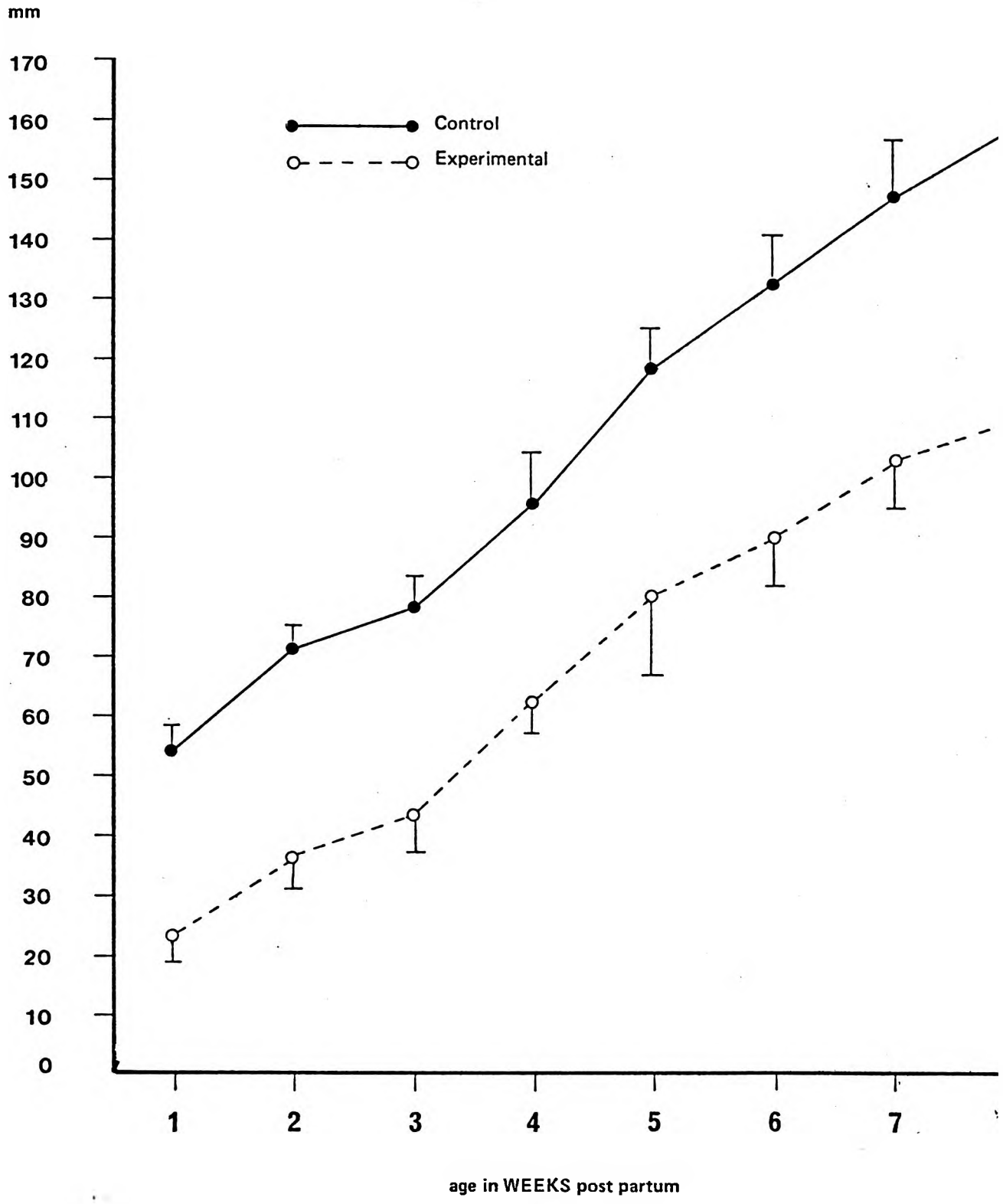


FIGURE 3.7 Graph showing mean tail length by age in weeks postpartum for offspring of rats exposed for first week. One standard deviation is indicated.

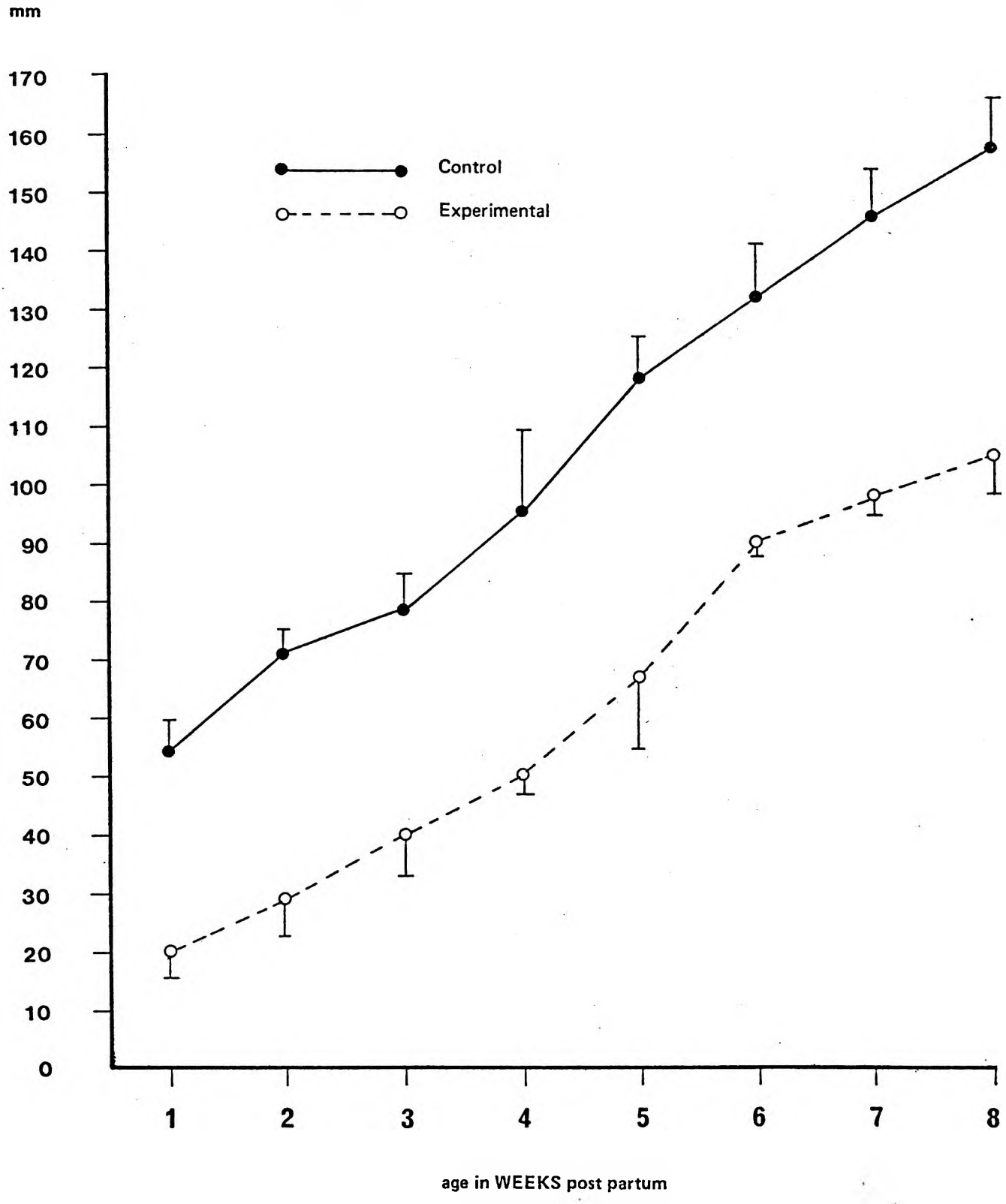


FIGURE 3.8 Graph showing mean tail length by age in weeks postpartum for offspring of rats (pooled results). One standard deviation is indicated.

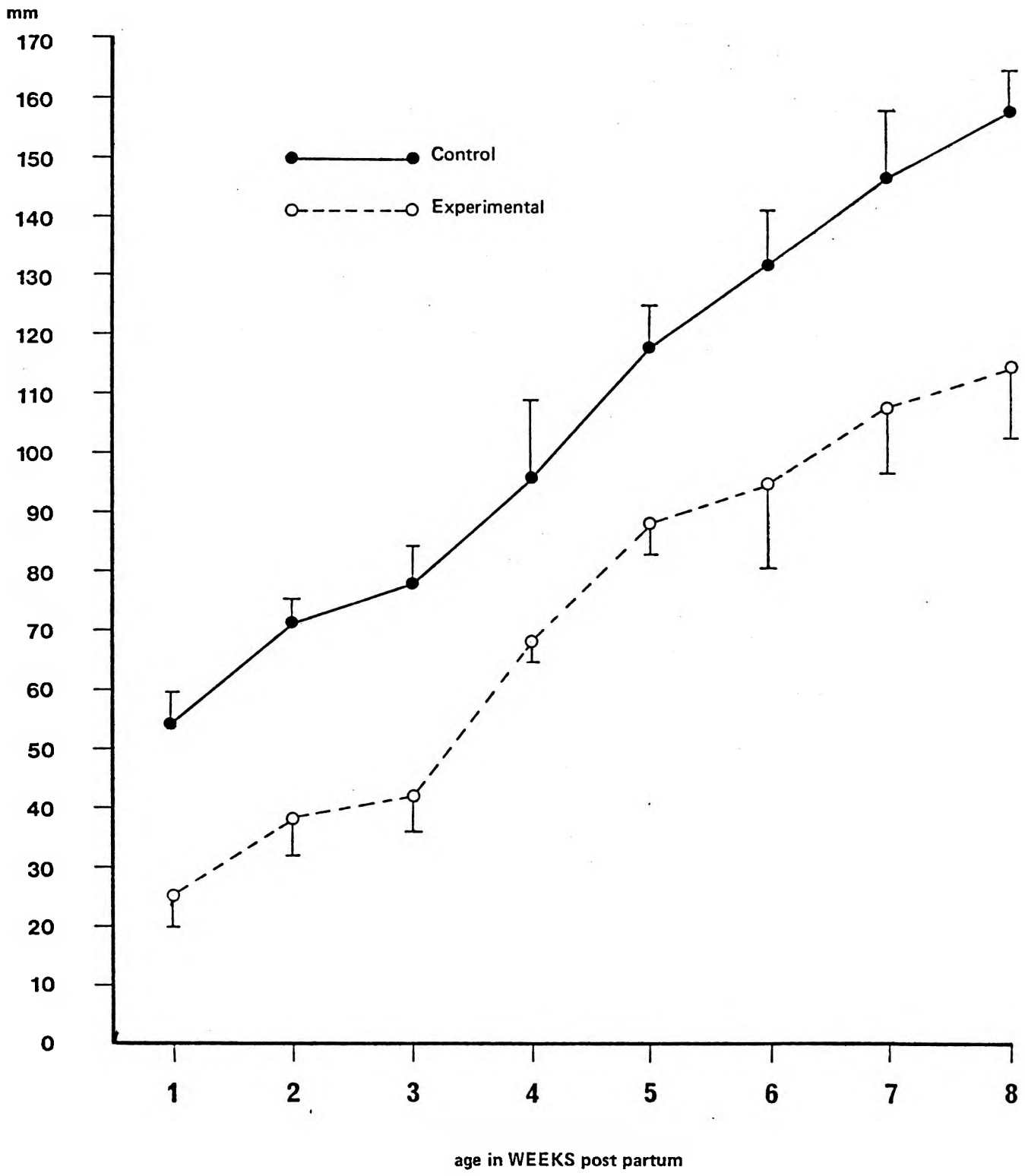


TABLE 3.4 Body length in millimeters of offspring

Weeks post partum	Control	Period exposed to N_2O								
		Pooled values for all N_2O-exposed groups			Three weeks			First and second weeks			First week		
	$\bar{x}$ ±	SD		$\bar{x}$ ±	SD		$\bar{x}$ ±	SD		$\bar{x}$ ±	SD		$\bar{x}$ ±	SD
1	59,5 ±	6,9		54,0 ±	4,9**		59,0 ±	4,4		56,3 ±	5,2		48,1 ±	5,3
2	79,5 ±	8,2		71,1 ±	6,5**		77,8 ±	5,9		76,7 ±	4,9		60,0 ±	8,7
3	88,2 ±	7,3		78,1 ±	16,0**		88,0 ±	4,2		77,8 ±	21,3		68,6 ±	9,2
4	108,8 ±	9,9		95,1 ±	9,8*		101,3 ±	8,3		107,0 ±	13,8		77,0 ±	7,4
5	138,7 ±	7,9		118,7 ±	18,7**		124,8 ±	16,6		131,5 ±	13,1		100,5 ±	26,4
6	148,6 ±	10,5		132,4 ±	15,0**		128,8 ±	11,8		142,6 ±	15,4		125,8 ±	17,8
7	156,7 ±	6,7		147,7 ±	8,0		150,6 ±	8,6		152,4 ±	9,1		140,0 ±	6,3
8	167,3 ±	5,6		159,2 ±	7,0		160,6 ±	8,2		162,0 ±	7,5		155,0 ±	5,5

P values for main group effects * P<0,05, ** P<001

FIGURE 3.9 Graph showing mean body length by age in weeks postpartum for offspring of rats exposed for all three weeks. One standard deviation is indicated.

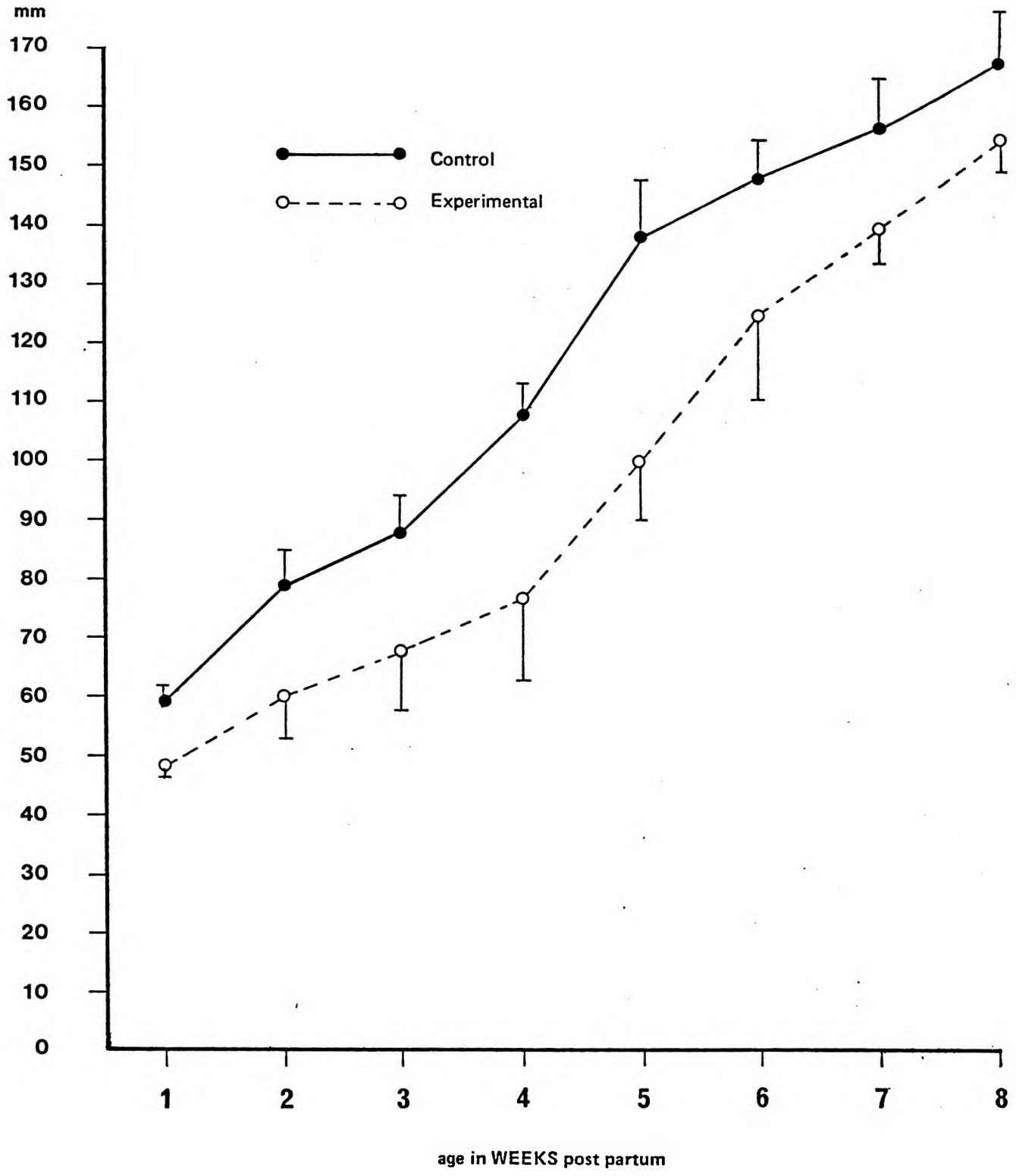


FIGURE 3.10 Graph showing mean body length by age in weeks postpartum for offspring of rats exposed for first and second weeks. One standard deviation is indicated.

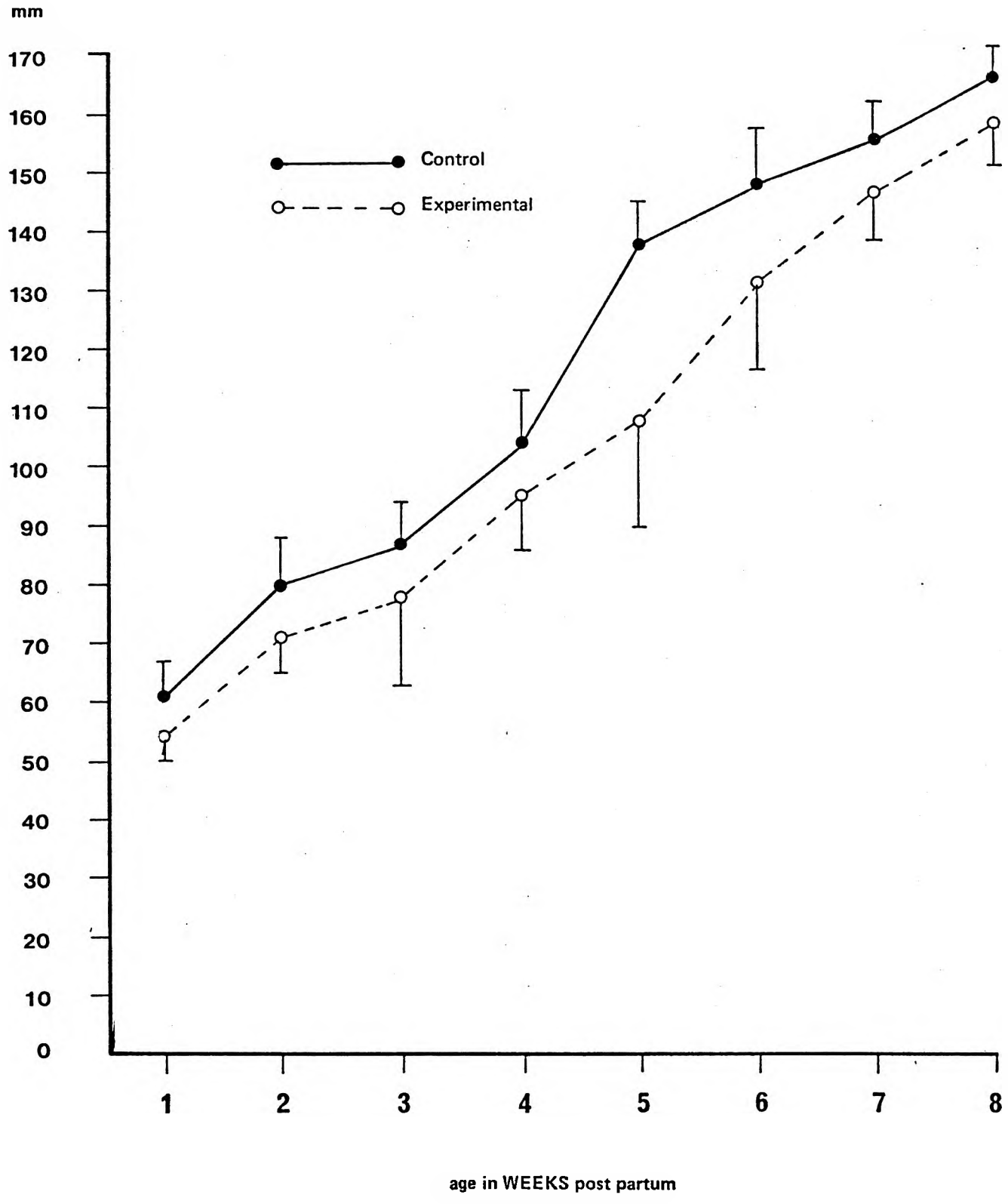


FIGURE 3.11 Graph showing mean body length by age in weeks postpartum for offspring of rats exposed for first week only. One standard deviation is indicated.

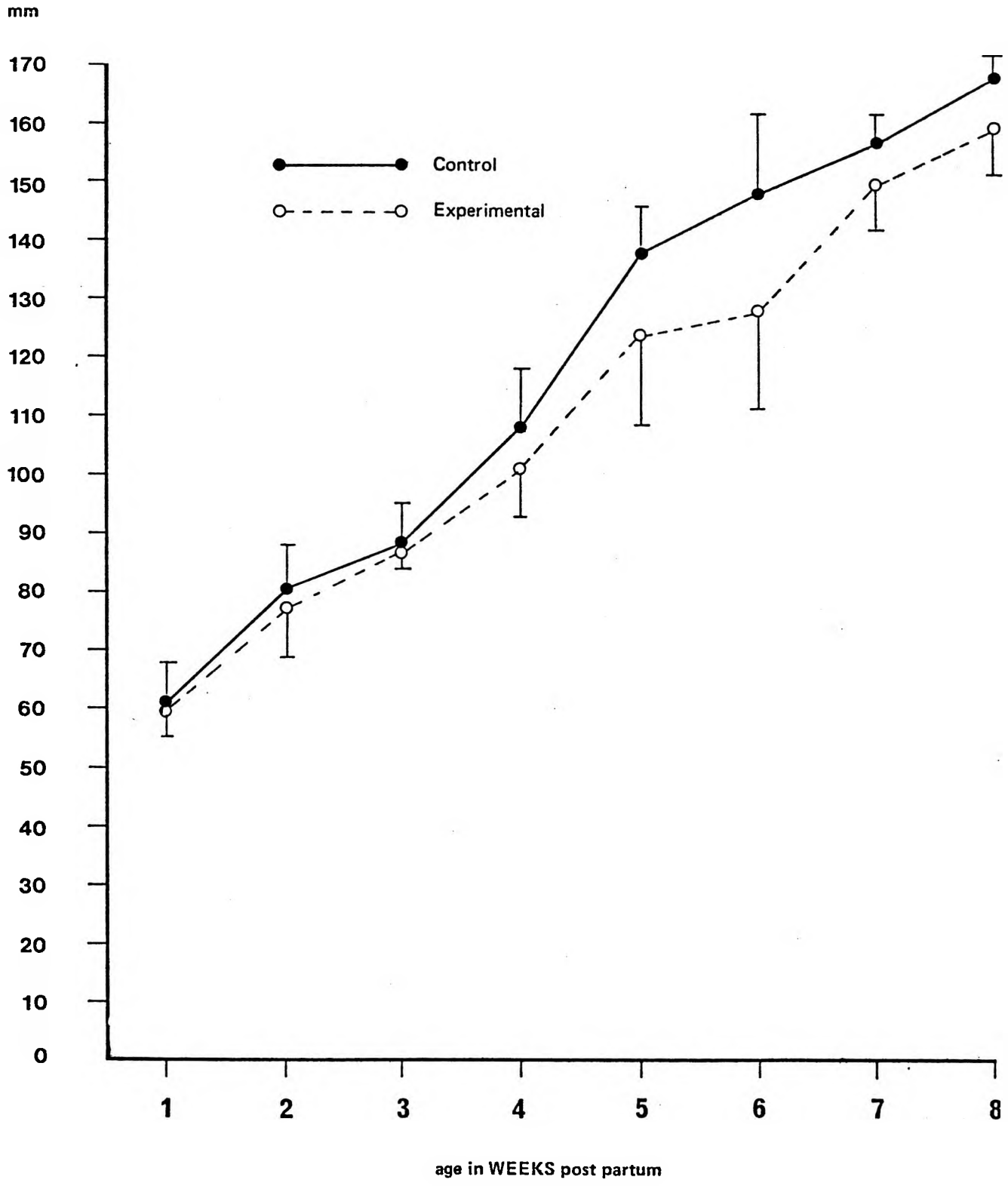
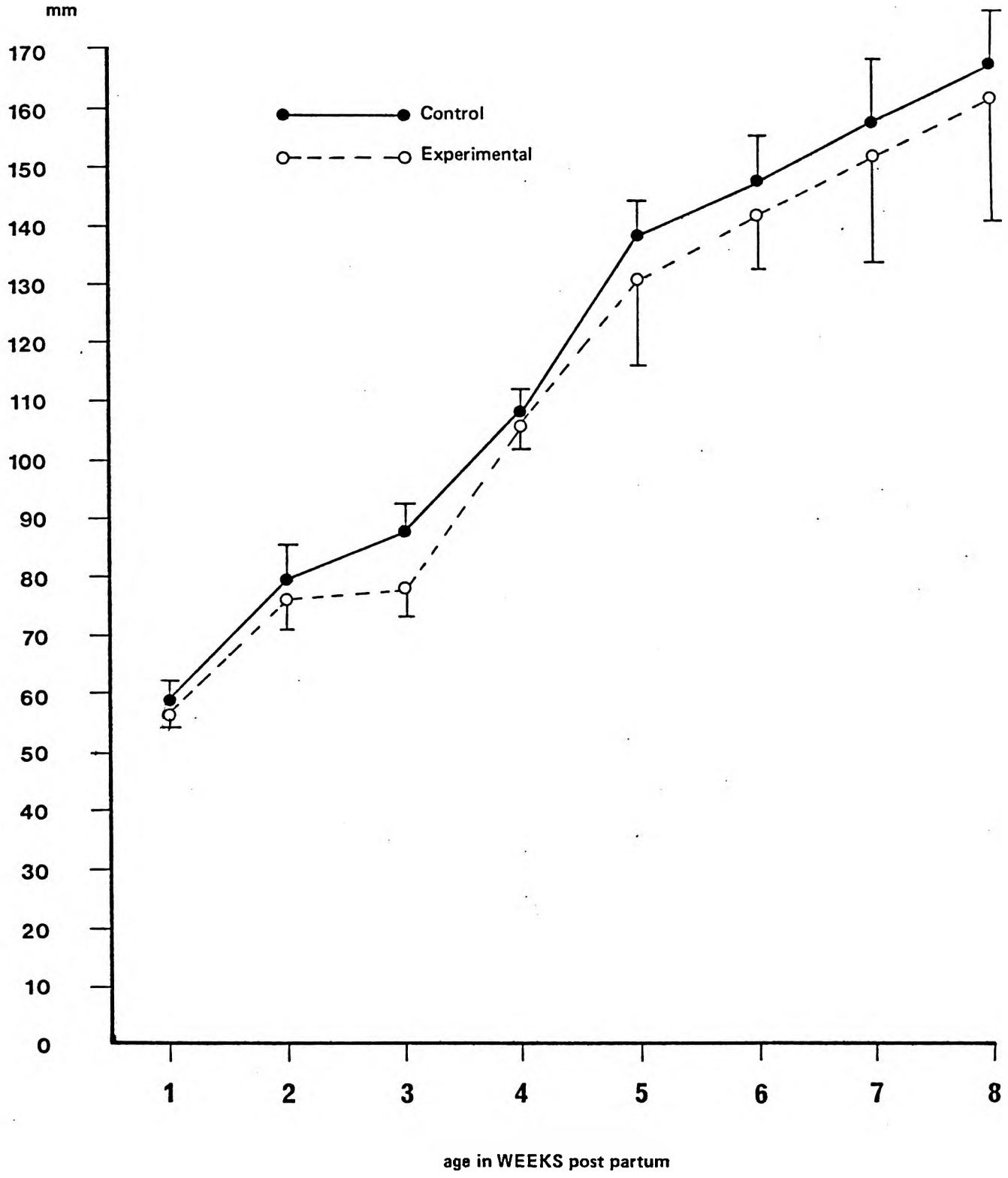


FIGURE 3.12 Graph showing mean body length by age in weeks postpartum for offspring of rats (pooled results). One standard deviation is indicated.



3.3.4 Cont.

There were significant differences between the body lengths of the rats exposed to the 1% nitrous oxide/air(V/V) as compared to the control group during each of the eight week periods except at week one. Here once again the offspring of rats exposed prenatally for their first week of gestation were smaller in size than those exposed for all three weeks and first and second weeks.

3.3.5 Main group effect

The group effect was examined using the modified formula:

i) S^2 pooled = S_p^2 -0,93 S_2^2 + 0,17 S_3^2

ii) Compute ratio F - $\frac{S_1^2}{S_p^2}$

which allows for the fact that the number of offspring in each litter is not constant. Table 3.5 shows the critical value for the main group effect. Body weight was significant throughout the first 8 weeks of growth. The tail length showed no main group effect for the first two weeks. The critical value for the body length was significant up to six weeks when there was no main group effect on body length for seven and eight weeks.

3.3.6 95% confidence intervals

Examination of the 95% confidence intervals of the data at the various periods revealed no particular pattern related to the timing of the exposure to nitrous oxide. On comparing the experimental groups it appeared that nitrous oxide had a greater effect on the rats exposed for only the first week of pregnancy. This was not constant throughout the results obtained over the eight week period of the study. For body weight this was found during the first, fourth and sixth weeks of growth, tail length during the first, third and fourth weeks of growth (Table 3.6)

TABLE 3.5 Table of Main Group Effects

Weeks	Body weight	Tail length	Body length
1	P<0,001	NS	P<0,001
2	P<0,001	NS	P<0,001
3	P<0,001	P<0,001	P<0,001
4	P<0,05	P<0,001	P<0,05
5	P<0,001	P<0,001	P<0,001
6	P<0,001	P<0,001	P<0,001
7	P<0,05	P<0,025	NS
8	P<0,001	P<0,001	NS

NS = Not Significant

TABLE 3.6 95% confidence intervals of results obtained for body weight, tail length and body length at the three exposure periods differing significantly one from another

Weeks of growth	Body weight	Tail length	Body length
1	First trimester	-	First trimester
2	-	-	-
3	-	First trimester	First trimester
4	First trimester	-	First trimester
5	-	First trimester	-
6	First trimester	-	-
7	-	-	-
8	-	First trimester	-

3.4 Discussion

Ramazotto, Katz and Cupiola (1975) examined the effect of short exposure to 50% (500 000 parts/10^6) nitrous oxide/air (V/V) on foetal death rate. They found a significant increase in foetal death rate in exposed rats. The findings, in this study in rats exposed during the first trimester and second trimester support Ramazotto et al's findings, however, three week exposure to one percent nitrous oxide, suprisingly did not produce a significant reduction in litter size. The explanation for the latter finding is obscure.

From the present study it was not possible to determine the reason for the decreased litter size. This will form part of the subsequent investigation (Chapter 4).

In this section of the dissertation it was concluded that intermittent exposure of gravid rats to a one percent concentration of nitrous oxide/air (V/V) significantly decreased litter size and the postnatal growth of the offspring. These findings showed too that the model system used was suitable for the production of reproductive system defects.

It was decided that further experimentation with continuous and intermittent exposure to lower concentrations of nitrous oxide/air (V/V) would be necessary to determine a level of concentration below which insignificant effects would occur.

CHAPTER 4

EFFECTS OF LOW CONCENTRATIONS OF NITROUS OXIDE/AIR ON DEVELOPING RAT FOETUSES (CONTINUOUS EXPOSURE)

4.1 Introduction

In Chapter 3, the nitrous oxide/air (V/V) was 1% (10 000 parts/10^6), a high concentration compared to the 25 parts/10^6 (V/V) maximum nitrous oxide pollution level recommended by the National Institute of Occupational Safety and Hygiene (Whitcher et al 1977). Review of the literature failed to reveal any evidence to support the choice of 25 parts/10^6 (V/V) consequently the investigations reported in this Chapter were undertaken to determine a concentration of nitrous oxide below which litter size would not be reduced, foetal abnormalities would not be seen and intra-uterine growth would not be retarded.

4.2 Materials and Methods

4.2.1 Choice of continuous exposure

Continuous exposure for the entire period of gestation was chosen to try and avoid the irregular effect produced by the intermittent exposure to 1% nitrous oxide/air (V/V) reported in Chapter 3.

4.2.2 Experimental design

Sixty rats were obtained from the South African Institute for Medical Research. These rats were randomly divided, using the same method as described in the previous Chapter, into five groups of 12 rats each - one control group and four experimental groups.

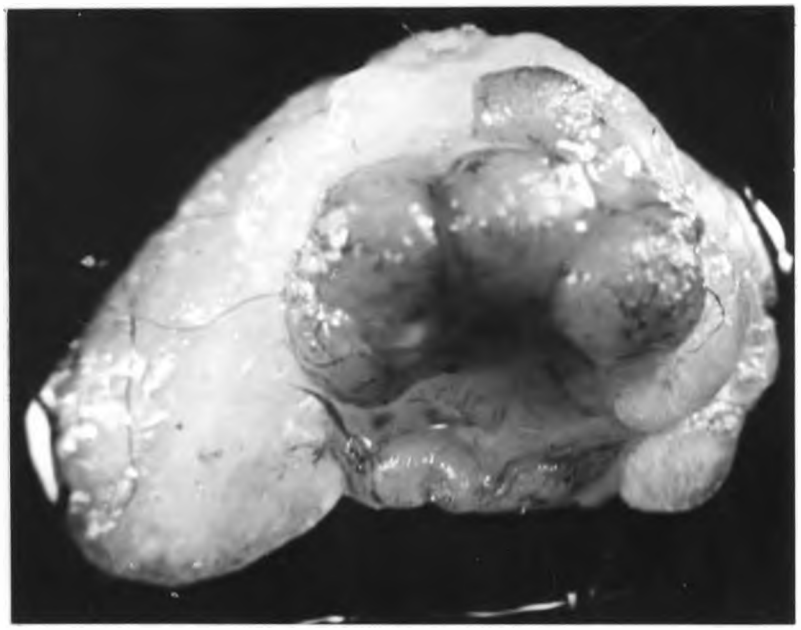
The gravid rats were placed into the environmental chambers for their entire gestation and exposed continuously to 0,5% (0,41kPa), 0,1% (0,08kPa), 0,05% (0,04kPa), 0,025% (0,02kPa) nitrous oxide/air (V/V).

All the rats were killed on day nineteen of their pregnancies by carbon dioxide administration in a lethal chamber. The gravid rats were then pinned down by their limbs, the abdominal viscera exposed by a midline incision, and a detailed examination of their ovaries and gravid uteri, using a dissecting microscope was carried out.

CHAPTER 4

4.2.2 Cont.

The numbers of corpora lutea of pregnancy were counted and compared with the numbers of foetuses present (Figure 4.1). When these numbers did not correspond the uterus was examined for placentation sites and embryonic remnants, signs which indicated where foetal resorption had occured. (Figure 4.2). The foetuses from each rat were counted, weighed, sexed and numbered for later investigation as discussed in Chapter 2, using the following format:



X12

Figure 4.1 Four corpora lutea of pregnancy in an ovary of a gravid rat.

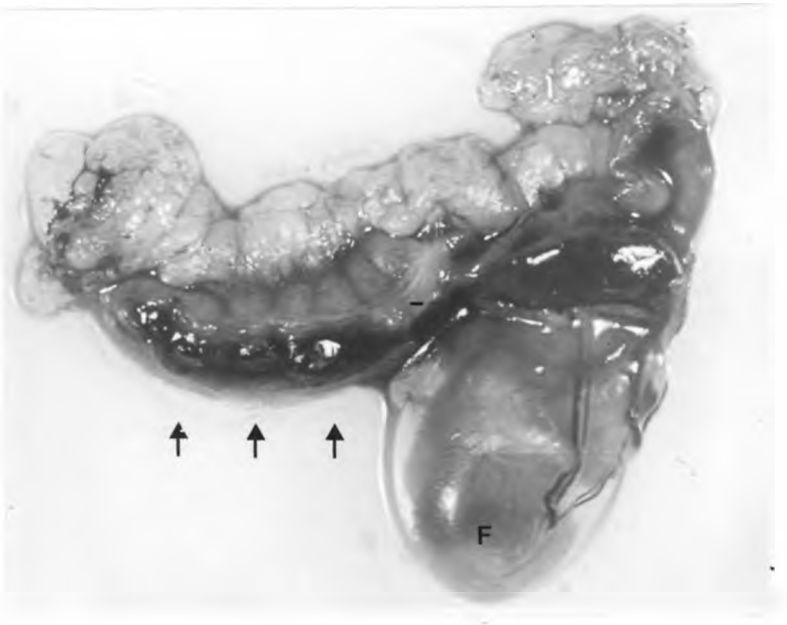


Figure 4.2 Uterus from a nitrous oxide exposed female showing one live foetus (f) and three foetuses undergoing resorption.

TERATOGENIC TESTING RECORD SHEET

SPECIES

NUMBER OF OFFSPRING

DRUG

ROUTE

DOSE

FREQUENCY

DATE OF FIRST DOSE

DATE OF FINAL DOSE

DATE OF CAESARIAN

CORPORA LUTEA COUNTED Left

Right

NUMBER OF RESORPTIONS

Positions of foetuses (left)	Sex	Weight	Malformations	Positions of foetuses (right)	Sex	Weight	Malformations

Number of live foetuses _____

Number of dead foetuses _____

Examination of the foetuses was undertaken using a dissecting microscope as described in Chapter 2, with reference to the following chart :

FOETAL EXAMINATION (using dissecting microscope)
DAM NUMBER
POSITION OF FOETUS
SEX OF FOETUS
FOETUS NUMBER

Palate												
Nasal cavities												
Eyes												
Ears												
Liver												
Kidneys												

Later crown-rump measurements done using a modified version of the method of van Rensburg (1976). Details of the measurement technique has been discussed in Chapter 2.

4.3 Results

4.3.1 Litter size

There were a total number of 501 foetuses produced by the five groups of rats - 120 in the control group, 66 in the group exposed to 0,5% nitrous oxide, 77 in the group exposed to 0,1% nitrous oxide/air (V/V), 118 in the group exposed to 0,05% nitrous oxide/air (V/V) and 120 in the group exposed to 0,025% nitrous oxide/air (V/V) (Table 4.1).

The litter size in the groups of rats exposed to 0,5% and 0,1% nitrous oxide/air (V/V) were almost half that in the control group and the other two experimental groups. This was significant at the one percent level.

TABLE 4.1 Litter size, crown-rump measurements and body weight of the control and experimental groups of rats exposed continually to N_2O .

	Litter size				Crown-rump measurements (mm)				Body weight (gm)			
	n	Range	Mean ± SD		n	Range	Mean ± SD		n	Range	Mean ± SD	
Compressed air												
Control	12	9-13	11,3 ± 1,4		120	32-50	44 ± 1,4		120	2,0-3,5	2,6 ± 1,38	
Nitrous oxide/air (V/V)												
0,5%	12	0-8	6,2 ± 5,0	p<0,001	66	25-30	29 ± 1,3	p<0,001	66	1,0-2,5	1,1 ± 2,8	p<0,001
0,1%	12	3-9	6,3 ± 4,0	p<0,001	77	30-45	35 ± 1,6	p<0,05	77	2,0-3,2	2,6 ± 1,4	NS
0,05%	12	9-13	11,0 ± 1,4	NS	118	32-50	43 ± 1,3	NS	118	2,0-3,4	2,6 ± 1,4	NS
0,025%	12	8-13	11,3 ± 1,3	NS	120	32-50	43 ± 1,4	NS	120	2,0-3,5	2,6 ± 1,4	NS

4.3.2 Foetal Resorption

Varying degrees of foetal resoprtion were recorded in ten of the twelve dams exposed to 0,5% nitrous oxide/air (V/V). Four of the dams showed death of all the foetuses (Table 4.2). Foetal resorption was also found to have occured in the group exposed to 0,1% nitrous oxide/air (V/V) where four of the twelve dams each showed resorption of one foetus. No foetal deaths or resorptions were found in the control dams or the dams exposed to 0,5% and 0,025% nitrous oxide/air (V/V). The chi-square test revealed a significant difference between the control and experimental groups that had foetal resorption ($p<0,001$).

4.3.3 Skeletal anomalies

The following chart was used for the skeletal examination detailed in the general materials and methods in Chapter 2.

Skeletal Examination

Cervical vertebrae												
Thoracic vertebrae												
Lumbar vertebrae												
Sacral vertebrae												
Ribs												
Other observations												

Skeletal anomalies were observed in 9% of the foetuses exposed to 0,5% nitrous oxide/air (V/V). The Fisher's exact probability test showed a significant p value of $p<0,05$ for skeletal malformations.

The anomalies found in this group were mainly in the form of skeletal malformations (Figures 4.3 & 4.4). The malformations of the ribs were associated with a scoliosis and kyphosis. These foetuses were much smaller in size than their litter mates and foetuses from the control group (Figures 4.5 - 4.6)

TABLE 4.2 Foetal information in experimental group exposed to 0,5% nitrous oxide/air (V/V)

	1	2	3	4	5	6	7	8	9	10	11	12
Live foetuses	7	9			13			1	10	10	5	11
Resorption sites	4	1	11	12	2	10	12		1			
Foetal weight - mean	1,44	1,33			1,80			1,28	1,46	1,66	1,82	2,05
(grams) S.D.	0,21	0,20			0,04			0,25	0,15	0,05	0,20	0,08
Crown-rump length - mean	29	28			30			29	28	28	28	29
(mm) S.D.	0,20	0,20			0,21			0,22	0,20	0,21	0,22	0,20
Live foetuses with Abnormalities		1			2			1	1	2		2
Number of live foetuses without abnormalities	7	8			11			10	10	8	5	9

X2.5

Figure 4.3 19 foetus from control group showing normal skeletal development.

X4.0

Figure 4.4 19 day nitrous oxide foetus showing stunted growth, rib malformations, a cervico-thoracic scoliosis (arrowed)

X3.3

Figure 4.5 Lateral view of a 19 day foetus from the nitrous oxide exposed group. It is smaller in size and shows abnormal ribs and vertebrae column.

Figure 4.6 Lateral view of a 19 day foetus from the control group.

4.3.4 Crown-rump measurements

Crown-rump measurements are shown in Table 4. These measurements of the foetuses exposed to 0,5% and 0,1% nitrous oxide/air(V/V) were smaller in size with a mean length of 29mm in the group exposed to 0,5% nitrous oxide/air (V/V) and 35mm in the group exposed to 0,1% nitrous oxide/air (V/V). The crown-rump measurements of the group exposed to 0,05% and 0,025% nitrous oxide/air (V/V) were not as significantly different to the measurement of those in the control group.

A two-way analysis of variance showed the difference between the groups exposed to 0,5% and 0,1% nitrous oxide and the control group to be highly significant ($p<0,001$). There was a less significant difference between the foetuses exposed to 0,05% and 0,025% nitrous oxide/air (V/V) and the control group ($p<0,05$).

4.4. Discussion

The reduction in litter size was associated with foetal death and resoprtion. This observation was confirmed by the fact that the numbers of corpora lutea of pregnancy recorded in the ovaries of rats with foetal resoprtion correspond exactly to the number of implantation sites found in the uterine horns. These results support the findings of Ramazotto et al (1975) and Vieira et al (1979) that nitrous oxide may be lethal to rat foetuses.

The reduced body weight observed in the foetuses which survived in the nitrous oxide exposed dams to 0,5% nitrous oxide/air (V/V) together with the significant reduction in the crown-rump measurements of same foetuses, complements the observation by Vieira et al in 1976 that prenatal growth of their offspring is reduced. This indicates that nitrous oxide retards growth in young offspring.

The 0,5% and 0,1% nitrous oxide/air (V/V) mixtures also produced abnormalities of the vertebrae and ribs. Similar observations were recorded by Fink et al in 1967 at a 50% nitrous oxide/air (V/V) mixture.

Results from the investigations reported in this Chapter indicate that the threshold value for continuous exposure, seems to lie between 0,1% (1 000 parts/10^6) and 0,05% (500 parts/10^6), concentrations considerably higher than 25 parts/10^6 nitrous oxide/air (V/V).

CHAPTER 5

EFFECT OF LOW CONCENTRATIONS OF NITROUS OXIDE/AIR ON DEVELOPING RAT FOETUSES (INTERMITTENT EXPOSURE)

5.1 Introduction

The results in Chapter 4 suggest that, following continuous exposure of gravid rats to nitrous oxide/air mixtures, the threshold value for reduced litter size, skeletal anomalies and prenatal growth retardation lies between 0,1% (1 000 parts/10^6) V/V) and 0,05% (500 parts/10^6 V/V) of nitrous oxide/air.

This chapter reports an experiment undertaken to determine the threshold value range for the above abnormalities following intermittent exposure to nitrous oxide/air mixtures.

5.2 Materials and Methods

Sixty rats were obtained from the South African Institute for Medical Research and divided into five groups of 12 rats each. One control group which was exposed to compressed air, the other four groups of rats being the experimental groups. Each group of 12 rats was exposed to 0,5% (5 000 parts/10^6), o,1% (1 000parts/10^6), 0,05% (500 parts/10^6) and 0,025% (250 parts/10^6) nitrous oxide/air (V/V) for six hours per day, five days per week for 3 weeks. The measuring technique and recording of data was the same as in the continuous exposure experiment in Chapter 4.

5.2.1 Experimental Design

The gravid rats were placed in the environmental chambers. The exposure period to the varying concentrations of nitrous oxide remained constant i.e. from 07h00 - 13h00 after which, the chambers were serviced. Monitoring of the environment was discussed in Chapter 2.

All the rats were killed on day 19 of their pregnancies and the same method of examination of the gravid uteri and foetuses as in the previous chapter was used. The rats were counted, numbered, sexed and measurements performed as described in Chapters 2 and 4.

5.3 Results

5.3.1 Litter Size

A total of 571 foetuses were produced by the control and four nitrous oxide/air exposed groups.

There were 120 foetuses in the control group, 98, 117, 117, 119 foetuses in the groups exposed to 0,5%, 0,1%, 0,05% and 0,025% nitrous oxide/air (V/V) respectively (Table 5.1). A significant difference in litter size existed only in the group of rats exposed to 0,5% nitrous oxide/air (V/V) as compared to the litter mates of the control and other experimental groups (Table 5.1).

5.3.2 Foetal Resorption

The gravid uteri of the rats were examined for foetal resorption and signs of embryonic remnants using a dissecting microscope as discussed in Chapter 2. The examination of the gravid uteri revealed no signs of foetal resorption in the control group or any of the groups exposed to the varying concentrations of nitrous oxide/air.

5.3.3 Skeletal Anomalies

The foetuses were examined for skeletal anomalies as described in Chapter 2. There was no evidence of any skeletal malformations in the control or the nitrous oxide/air exposed groups.

5.3.4 Crown-rump measurements

Crown-rump measurements are shown in Table 5.1. The foetuses of the control group had a mean crown-rump measurement of 44mm, with a range of 32 - 50mm. In the nitrous oxide exposed group the means were as follows, o,5% nitrous oxide/air (V/V) with a mean of 38mm, range between 29 - 50mm, o,1% nitrous oxide/air (V/V) had a mean of 40mm, range between 30 - 50mm, 0,05% nitrous oxide/air (V/V) with a mean of 42mm, range between 31 - 50mm and 0,025% nitrous oxide/air (V/V) with a mean of 42mm and range between 32 - 50mm. Once again, there was no significant difference in the nitrous oxide and control dams.

TABLE 5.1 Litter size, crown-rump measurements and body weight of the control and experimental groups of rats exposed intermittently to nitrous oxide

	Litter size				Crown-rump measurements(mm)				Body weight(gms)			
	n	Range	Mean ± SD		n	Range	Mean ± SD		n	Range	Mean ± SD	
Compressed Air												
Control	12	9-13	11,3 ± 1,4		120	32-50	44 ± 1,4		120	2,0-3,5	2,6 ± 1,38	
Nitrous oxide												
0,5%	12	6-10	7,0 ± 2,3	p<0,001	98	29-50	38 ± 1,2	NS	98	2,0-3,1	2,2 ± 1,2	NS
0,1%	12	8-13	10,0 ± 1,2	NS	117	30-50	40 ± 1,3	NS	117	2,0-3,2	2,4 ± 1,2	NS
0,05%	12	8-13	11,0 ± 1,3	NS	117	31-50	42 ± 1,2	NS	117	2,0-3,2	2,5 ± 1,3	NS
0,025%	12	9-13	11,2 ± 1,3	NS	119	32-50	42 ± 1,4	NS	119	2,0-3,5	2,6 ± 1,4	NS

5.3.5 Body weight

Body weight of the foetuses showed a similar pattern of non-significance. The control dams had a mean body weight of 2,6 grams, with a range of 2,0 - 3,5 grams. The 0,5%, 0,1%, 0,05% and 0,025% nitrous oxide/air (V/V) exposed foetuses had a mean body weight of 2,2 grams (range 2,0 - 3,1 grams) 2,4 grams (range 2,0 - 3,2 grams), 2,5 grams (range 2,0 - 3,2 grams and 2,6 grams (range 2,0 - 3,5 grams) respectively.

5.4 Discussion

As had been expected the threshold limit value was raised to between 0,5% and 0,1% nitrous oxide/air (V/V) thus indicating that gravid rats exposed intermittently to the lowe concentrations of nitrous oxide investigated in this dissertation produced little or no effect on the prenatal development of the offspring, but nevertheless a significant reduction in litter size was still apparent.

5.5 Overall Conclusions

The studies reported in this dissertation have shown:

1. that the experimental model system used is suitable for the examination of the effects of trace concentrations of nitrous oxide on gravid laboratory rats.
2. that 1% nitrous oxide in air (V/V) inhaled intermittently by gravid laboratory rats produced statistically significant reductions in litter size and postnatal growth of offspring.
3. that following continuous inhalation of 0,5% 0,1%, 0,05% and 0,025% nitrous oxide/air (V/V) by gravid laboratory rats, the threshold value for reproductive system defects, appears to lie between 0,1% and 0,05%.
4. that, following intermittent inhalation of 0,5% 0,1%, 0,05% and 0,025% nitrous oxide in air (V/V) by gravid laboratory rats, the threshold value for reproductive system defects, appears to lie between 0,5% and 0,1%.

5.5 Overall Conclusions

5. that, 25 parts/10^6 maximal pollution level for nitrous oxide suggested by NIOSH seems unrealistic and that a higher level may be reasonable.
6. that the reproductive defects produced support the concept of reduced exposure of operating room and dental surgery staff to nitrous oxide, although the level for humans remains to be determined.

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