THE PHYSIOLOGY AND ECOLOGY OF THE EGGS OF THE PLEURODIRAN TORTOISE *EMYDURA MACQUARII* (GRAY), 1831

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

JUNE 1983

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SUMMARY

The eggs of the Pleurodiran short-necked tortoise, *Emydura macquarii*, are intermediate between the hard-shelled and hard, expansible-shelled eggs studied to date. Therefore aspects of the physiology and ecology of the eggs of *E. macquarii* were studied and compared to Cryptodiran species from other parts of the world. Measurements of physical characteristics of the eggs, including their mass, length, breadth, surface area, shell and membrane thicknesses, ultrastructure of the shell and membranes, and of the pores in the shell, were made to help describe and enable interpretations of experiments on the gas and water relations of the embryos.

Eggs of *E. macquarii* are 36.11 mm long x 22.02 mm wide (mean) and the mean mass is 10.423 g. There are positive semilogarithmic correlations between egg length and breadth, length and mass, and breadth and mass. Elongation is constant over the range of egg sizes. Egg mass is positively correlated with female size supporting the suggestion that egg breadth is limited by the dimensions of the mother's pelvis. Hatchlings are 48.06% of the fresh egg mass regardless of egg size or the conditions experienced during incubation. The egg is made up of yolk (37%), albumen (47%) and shell (14%). The yolk is 68% water, albumen 95% water and shell 22% water. The water content of fresh eggs and hatchlings is similar. The water content of embryos falls from 95% to 77% between the middle of the incubation and hatching. In unhatched eggs the shell is thickest around the equatorial plane (0.191 mm) and thinnest at the poles (0.155 mm). Shell from the equatorial plane is thinner (0.177 mm) and at the poles thicker (0.166 mm) in hatched eggs than unhatched ones. Membranes from the equator are as thick (0.06 mm) as those from poles of the egg.

As the structure of the shell is similar to that of other species of chelonians with well organised shells, the process of shell formation is the same. Pores are Types 1a and 3a(ii) of Board *et al* (1977) and are concentrated in the equatorial regions of the shell. There is no external cuticle. Counts of the number of pores per egg ranged from 103 to 17,720 ($\bar{x}$ = 3,673) and the mean minimum diameter is 18.5 μ. Lines of intense dissolution on the inner surface of the shell of hatched eggs are assumed to be adjacent to blood vessels in the chorioallantois. The shell membranes are composed of five layers of fibres of different diameter.

The pattern of O_2 consumption ($\dot{V}_{O_2}$) of eggs of *E. macquarii* increases exponentially during the first 80% of the incubation, peaking at 87% of the way through the incubation and then falling to 61 - 82% of the peak value before hatching. There is a slight rise in the rate of respiration at hatching due to the efforts of the hatchling to free itself from the egg. Equal amounts of O_2 are consumed during incubation at 25°C and 30°C (580 ml), and mass-specific $\dot{V}_{O_2}$ is equal at both temperatures (110 $ml.g^{-1}$ of hatchling mass). Embryonic growth, indicated by mass, reflects the change in $\dot{V}_{O_2}$, increasing exponentially for the first 70 - 80% of the incubation and then slowing prior to hatching. $\dot{V}_{O_2}$ is related to embryonic mass (corrected to the water content of hatchlings) raised to the power 0.862, similar to the exponent for avian embryos (0.92), which supports the idea that the cost of biosynthesis in embryos raises this exponent above that for adult animals in general (0.75). Respiratory exchange ratio (RE) of 0.61 is not significantly different at 25° and 30°C. Most lipid (94.2%) found in fresh eggs is contained in the yolk; lipid accounts for 76.7% of the energy used during incubation. Mean Q_{10} is 2.87.

The different patterns of embryonic respiration shown by chelonians may allow synchronous hatching, as in some birds. Thus deeply buried

eggs, which experience essentially the same temperatures in any one clutch during incubation, grow at the same rate and hatch simultaneously, have an exponential pattern of embryonic respiration. In contrast, eggs at intermediate depths, e.g. *E. macquarii*, experience a range of temperatures in different parts of the nest and therefore develop at different rates. The period of time between the peak of respiration and hatching may be varied in different individuals to allow the synchronous hatching of eggs at slightly different stages of development.

In natural nests in the field P_{O_2} is depressed by about 10 torr and P_{CO_2} elevated by about 4 torr over controls just prior to hatching. The maximum ΔP_{O_2} and ΔP_{CO_2} across the dry shell, calculated from weight loss data over silica gel at constant temperature are 2.1 and 1.6 torr and from conductance values across the normally partially hydrated shell and membranes measured directly are 19.0 and 7.5 torr. The shell membranes are a more significant barrier to the diffusion of O_2 and CO_2 than is the dry shell. However both shell and membranes dry out regionally during development resulting in the formation of a small white patch on the uppermost surface of the egg within 30 h of being laid. This patch grows to eventually cover the whole egg surface in some eggs, thus facilitating gas exchange through the eggshell in advance of the requirements of the embryo. The conductance of shell and membranes to O_2 and CO_2 increases with the area of the white patch reaching 2.6 ± 1.1 $cm^3.day^{-1}.torr^{-1}$ and 4.7 ± 1.3 $cm^3.day^{-1}.torr^{-1}$ at $30^{\circ}C$ for eggs with the white patch covering the whole shell.

E. macquarii does not appear to select nesting sites in the field on the basis of the hydric properties of the soil. The tolerance of the eggs to widely different hydric conditions during incubation reflects this. Eggs were incubated completely buried in, half buried in, and sus-

pended above substrates ranging in water potential from -50 kPa to -3,550 kPa. Eggs buried at -50, -110 and -220 kPa and those half buried at -220 kPa had reduced hatching rates; all other treatments recorded high hatching success. All eggs lose weight for the first third of the incubation regardless of the water potential of the substrate. Eggs in the wettest substrates gain water at an increasing rate until hatching, those on drier substrates lose water at a constant rate. All eggs suspended above the substrate sustain a net loss of water. The shells of eggs that gain water crack to accomodate it. Eggs that successfully hatched ranged from those that lost 27.5% of their fresh egg mass to those that gained 32.0%. An experiment using fluorocein and tritiated water confirmed that eggs contacting the substrate absorb liquid water from that substrate but liquid water does not flow between eggs. Water potential measurements of egg contents indicate that water is drawn into the egg against a water potential gradient of up to 320 kPa but it is not known how this occurs. Desiccating conditions experienced during incubation do not result in abnormal hatchlings in *E. macquarii* as they do in other species. The term cleidoic is discussed with regard to the degree of water uptake by non-cleidoic eggs. Eggs such as *E. macquarii*, which imbibe water under certain conditions, but the effect of this does not influence the size of the hatchlings, are termed "facultative cleidoic" eggs.

Nests of *E. macquarii* in the field experience temperatures less severe than the soil surface but still experience diurnal temperature fluctuations up to 10°C. The temperature of nests rises throughout the incubation period as the summer progresses. Metabolic heat does not measurably raise nest temperatures. Incubation time in the laboratory is inversely proportional to incubation temperature. Incubation time in the field is influenced by the degree of shading of the nest. Adult sex ratio is significantly biased towards females. Sex determination in

E. macquarii is independent of incubation temperature, with a 1 ♂:1 ♀ sex ratio at incubation temperatures of 20, 25, 26, 28, 30 and 32°C. Temperature-dependent sex determination may be an adaptation that ensures outbreeding and has its greatest advantage in small discrete populations with relatively high rates of predation on the eggs.

On the River Murray in South Australia the introduced fox, *Vulpes vulpes*, takes 93% of chelid tortoise nests and other predators (water rats, goannas and ravens) take a further 2.7%. Death of eggs due to other causes is rare. The size structure of the population of *E. macquarii* in the Murray was compared to that of a closely related species from the Cooper Creek, which lives in the virtual absence of foxes, but which has essentially the same endemic nest predators that occur on the Murray. There are a significantly higher number of juveniles and young adults in the Cooper Creek than the Murray population. There appears to be a significant reduction in juvenile recruitment into the Murray population of *E. macquarii* resulting in a shift in the age structure of the population towards old individuals resulting in a gradual decline in the size of the population.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

MICHAEL BADEN THOMPSON

ACKNOWLEDGEMENTS

Many people volunteered their services and time freely to help me with this study. I am indebted to them all. For help in the field I am particularly grateful to J. Bannear, K. Bird, D. Booth, D. Carter, P. Christy, P. Cockerham, T. Hudson, M. Keck, B. Mitchel, D. Papps, S. Richards, T. Sim, J. Thompson and K. Turner. Specimens of dead tortoises were collected from the Cooper Creek by L. and J. Peddler. The South Australian Departments of Fisheries and Environment and Planning issued permits to net tortoises in the Murray and Cooper and lent me equipment to do so. For help in the laboratory I would like to thank D. Booth, B. Carson, P. Christy and J. Gibbs. Dr. B. Howard of the Department of Animal Physiology at the Waite Agricultural Research Institute extracted water from egg fractions and determined the tritium content by liquid scintillation; Mr. J. Forrest of the C.S.I.R.O. Division of Soils measured the water potential of soil samples by ceramic plate extraction. Several University Departments helped including the Department of Botany (loan of Dew Point Microvoltmeter), Physiology Department (loan of Medical Gas Analyser), Dental School (X-rays) and most importantly the University's glass blower. The German papers were kindly translated by M. Keck.

Mr. J. Bannear of Loveday showed me the nesting sites at Lake Bonney, taught me how to locate nests and allowed me free use of his tortoise breeding facility. Without him the practical side of my study would still be in the dark ages and I would like to record my appreciation of his help and enthusiasm. The nesting site at Monteith was shown to me by Mr. T. Hudson and at Milang by Mr. T. Sim.

A number of people were stimulating sounding-blocks for many of my ideas. In particular were P. Christy, D. Papps, D. Booth, D. Carter, C. and D. Vleck and J. Wright. My supervisor, Dr. Roger Seymour was the ultimate sounding-block. Although he allowed me a free hand to determine my own destiny, acting in his self-titled role as "devils advocate" he kept me from straying too far from reality. I am grateful to Roger Seymour, Alice Wells and Keith Walker for reading and critising the drafts of this thesis.

Mr. P. Kempster helped me with the final preparation of the plates in the thesis and the manuscript was expertly typed by Mrs. P. Jowitt. Mr. B. Thompson helped with photocopying.

Finally I would like to thank my wife Jo, who supported me in every way possible for the "duration".

This work was funded by a University of Adelaide Research Grants Scholarship for Postgraduate Research and by two grants from the Reserves Advisory Committee of the former South Australian Department for the Environment.

1

GENERAL INTRODUCTION

Emydura macquarii (Gray) is a freshwater tortoise common in the Murray-Darling system of south-eastern Australia (Goode, 1967; Cann, 1978; Cogger, 1979). It is a Pleurodire (suborder Pleurodira of Romer (1956); infraorder Pleurodira of Gaffney (1975)) and belongs to the Chelidae, the dominant freshwater chelonian family in Australia. The other extant suborder of the Chelonia, the Cryptodira, is represented in Australia by the marine turtles and one freshwater Carettochelyid.

The dichotomy of the Pleurodira and Cryptodira occurred very early in the history of turtles, probably prior to the late Triassic (Gaffney, 1975). The Pleurodira are regarded by Romer (1956) as a primitive group, and the Cryptodira as advanced. The two groups are widely divergent morphologically. Their most obvious external difference is the method of retraction of the head: in the Pleurodira the neck is withdrawn laterally, whereas in the Cryptodira it is withdrawn with a vertical flexure. There are many skeletal differences including the fusion of the pelvis to the carapace and plastron, the occurrence of transverse processes on the cervical vertebrae, the distance between the cervical postzygapophyses, development of sacral ribs, fusion of atlas and odontoid and many characters of the skull (Romer, 1956; Gaffney, 1975).

Today the Pleurodira occur only in the southern continents, with the family Chelidae being restricted to Australia, New Guinea and South America (Goode, 1967). The Australian and New Guinea genera show close affinities with South American genera (Gaffney, 1977).

All chelonians are oviparous (G. Packard *et al.*, 1977a) their eggs range from soft-shelled to hard-shelled (Ewert, 1979; M. Packard *et al.*, 1982a). The eggs are incubated in a chamber constructed in the soil by the mother. The chamber is excavated with the hind limbs in a manner

that is similar throughout the order. Because many species of chelonians are aquatic they must emerge from the water to deposit eggs in nests constructed in beaches and banks. Once nesting is completed the mother returns to the water and the eggs incubate without parental care. In the period from oviposition to hatchling emergence, chelonians are vulnerable to changes in environmental conditions and to predation. Conditions of incubation, such as temperature and humidity, experienced by the eggs are determined by the environment, yet causes of death other than predation are rare in *E. macquarii*. This implies that either the eggs are tolerant of a wide range of environmental conditions or the female constructs the nest in a manner, place and time that optimises the chances of successful incubation. To understand the adaptations that enhance the hatching success of eggs it is necessary to measure the physical conditions experienced by eggs during natural incubation, compare them to the range of conditions existing in the environment and to measure the tolerances of eggs to that range of environmental conditions.

Much of the information available on the eggs and embryos of reptiles is anecdotal. It lacks the specific detail needed for comparisons of the responses of eggs from different populations or species to particular environmental conditions and for predictions of their behaviour in the field (G. Packard *et al.*, 1977a). The lack of information on the eggs of reptiles contrasts strongly with that on birds, in particular the domestic hen (e.g. Visschedijk, 1968a,b,c; Tyler, 1969; Wangensteen & Rahn, 1970/71; Wangensteen *et al.*, 1970/71; Tazawa *et al.*, 1971; Fujii, 1974; Simkiss, 1980a). The eggs of many other species of birds have recently been studied (e.g. Rahn *et al.*, 1976; Board *et al.*, 1977; C. Vleck *et al.*, 1980; D. Vleck *et al.*, 1980) and provide a useful base-line with which reptilian eggs may be compared.

With the exception of limited ecological and physiological studies on common species (Parmenter, 1976; Chessman, 1978) and the rare western swamp tortoise, *Pseudemydura umbrina* (Burbidge, 1967, 1981) the Chelidae of Australia (15 species according to Cogger, 1979) are still little known. Most of the available information on chelonians and their eggs comes from North American and European cryptodiran species (G. Packard *et al.*, 1977a; Harless & Morlock, 1979; M. Packard *et al.*, 1982a) and there is no detailed information on the eggs of Pleurodiran species with which comparisons can be made.

The eggs of chelonians have been categorized into three groups (Ewert, 1979): hard-shelled and soft-shelled eggs and an intermediate group that are hard when laid but the shell cracks as the egg absorbs water and expands during incubation. These eggs are termed hard, expansible-shelled. The eggs of the Australian chelid tortoise, *Chelodina longicollis*, have been identified as a rare intermediate between hard, expansible-shelled and hard-shelled eggs because the hard shell fractures into small flakes midway through the incubation (Ewert, 1979). Eggs of *C. longicollis* that I examined are structurally similar to those of *E. macquarii* and they absorb water in a similar manner during incubation. Hence a study of the eggs of *E. macquarii* is significant because, not only are they Pleurodiran, but they may represent an intermediate in the range of egg types studied to date.

Since the inception of this investigation there has been a rapid increase in knowledge of the structure and water relations of chelonian eggs (M. Packard *et al.*, 1982a), the respiration of chelonian embryos (Ackerman, 1981b) and the effects of incubation temperature on sex determination (Bull, 1980). In all cases these studies have dealt with crypto-

diran species. This study is the first to investigate these aspects in a pleurodiran species.

Detailed ultrastructural analyses of the shells of chelonian eggs show structural differences in the calcareous part of the shell of eggs in the three categories. The shell of hard-shelled eggs is thick relative to the underlying shell membranes and is composed of tightly fitting calcareous units whereas the shell of soft-shelled eggs is about as thick as the membranes and is composed of poorly defined shell units loosely organised into an open matrix; hard, expansible-shelled eggs are intermediate (M. Packard & Packard, 1979; M. Packard *et al.*, 1982a). The structure of the eggshell of *E. macquarii* was studied for comparison with other species because their intermediacy between hard-shelled and hard, expansible-shelled eggs could be verified in this way.

Changes in concentrations of O_2 and CO_2 in the nests of marine turtles during incubation are similar to those in the air cell of a single avian egg (Ackerman, 1977). Because the shell of these marine turtle eggs have a high conductance to gases (Ackerman & Prange, 1972), the subshell gas concentrations are close to those of the nest atmosphere. It was suggested that the gas concentrations in the nests of turtles and the air cell of avian eggs may serve an important physiological function, such as stimulating the embryo to pip the shell, in both groups of vertebrates (Ackerman, 1977). Therefore I calculated the gas concentrations experienced by embryonic *E. macquarii* and examined how this may change throughout incubation. To do so I measured the tensions of O_2 and CO_2 in natural nests in the field throughout the incubation to establish the nest environment of the eggs. I measured the rate of respiration of eggs at two temperatures in the laboratory and temperatures in nests in the field throughout the incubation because the rate of respiration is re-

lated to temperature. The respiration measurements showed when the maximum rate of O_2 consumption occurs during incubation. Measurements of respiration at two temperatures allows the calculation of Q_{10} which is then used to calculate maximum O_2 consumption at maximum nest temperatures. Measurements of the respiratory exchange ratio then allow calculations of maximum CO_2 production. To calculate the changes in gas tensions between the nest atmosphere and the embryo, i.e. across the eggshell and membranes, I measured the conductance of the shell and shell plus membranes in the laboratory. Combining the two values at the peak of embryonic respiration enabled me to calculate the gas tensions experienced by embryonic *E. macquarii* prior to hatching to test the hypothesis that gas tensions experienced by *E. macquarii* are similar to those in birds and marine turtles and that they may therefore serve a necessary physiological function.

The pattern of change of O_2 consumption throughout incubation was measured in embryonic *E. macquarii*. Patterns of embryonic respiration have been described for other reptiles (Ackerman, 1981b; Hoyt & Albers, in press) and birds (C. Vleck *et al.*, 1979, 1980; D. Vleck *et al.*, 1980). In marine turtles, snakes and altricial birds the rate of respiration increases throughout the whole incubation, whereas in some chelonians and most precocial birds the rate of respiration increases exponentially for the first 80% of the incubation and then slows prior to hatching. In other precocial birds this pattern is modified and the rate of respiration peaks about 80% of the way through the incubation and then falls before hatching. In birds the patterns of respiration are related to the development of the embryos (C. Vleck *et al.*, 1980) and the modification shown by some precocial birds allows synchronous hatching. The patterns of respiration during incubation in different chelonians were compared and related to synchrony of hatching.

An important physical variable influencing chelonian embryos is the hydric conditions of incubation. Because the nest chamber remains largely free of soil (Cagle, 1950; Ackerman, 1977; G. Packard *et al.*, 1977a, 1979a, 1981c), in most nests the eggs on the outside of the clutch contact the substrate and those in the middle contact only other eggs. The effect of different hydric conditions during incubation on eggs contacting the substrate and those held away from the substrate has been studied in hard-shelled (G. Packard *et al.*, 1979a, 1981c), soft-shelled (Tracy *et al.*, 1978; G. Packard *et al.*, 1980, 1981a,b,d) and hard expansible-shelled eggs (G. Packard *et al.*, 1982). The soft-shelled eggs readily take up water and expand in favourable (wet) incubation conditions and the hatchlings emerging from eggs incubated in moist conditions are larger than those incubated in dryer conditions. In contrast the hard-shelled eggs are unable to expand because of the rigid shell and all eggs experience a net loss of water during incubation. The hydric conditions of incubation do not influence the size of the hatchlings. Hard, expansible-shelled eggs behave in an intermediate manner, with weight changes of eggs being influenced by substrate water potential and hatchlings size being only partly influenced by substrate water potential. Similar experiments were carried out on the eggs of *E. macquarii* for comparison. The response to available water during incubation was expected to be intermediate if the eggs are intermediate between hard and hard, expansible-shelled eggs.

The possibility that the sex of hatchlings is determined by the temperature of incubation, as it is in most other chelonians studied (Bull, 1980; Pieau, 1982), was tested in *E. macquarii*. The determination of sex by incubation temperature has important implications for the management of a species, particularly rare species where eggs are often artificially incubated and the hatchlings given a head start (Mrosousky & Yntema, 1980).

The detailed study of the physical conditions of naturally incubating eggs, and the physiological tolerances of those eggs, may be useful in decisions regarding the conservation management of a species. However, provision of optimal physical conditions for nesting and incubation may not be adequate protection for a species if the rate of predation on the eggs is too high. Hence I studied the effect that predation on nests may be having on populations of *E. macquarii* in the River Murray. This is particularly important in this case because the major predator, the red fox, is introduced, so the rates of predation experienced by the population may be different from those prior to the introduction of foxes.

Each chapter in this thesis discusses the topics outlined above (physical characteristics, metabolism, gas relations, water relations, effects of temperature and predation) and is written to stand alone as an experimental analysis of the data associated with the question. Apart from the main aim, in each chapter a number of associated questions arise, are tested and discussed, each being complimentary to the main question. The thesis concludes with a short discussion that deals, in part, with the implications of the results of this study for possible management of populations of *E. macquarii*.

2

TERMINOLOGY AND GENERAL METHODS

The following chapters describe the results of largely separate investigations. Terminology throughout is consistent but some definition of the terms used is warranted. Some techniques were used in more than one chapter so they are described here for the sake of brevity.

2.1 TERMINOLOGY

Common Name. In Australia the Chelonia is represented by six marine turtles, the pig-nosed turtle (*Carettochelys insculpta*) and the fresh-water Chelidae (Cogger, 1979). *Carettochelys* is a relatively recent discovery on this continent. In the absence of any true terrestrial tortoises (Testudinidae) the Chelidae are commonly known as "tortoises", because they have clawed feet, to distinguish them from marine "turtles" and *Carettochelys*, which have flippers. Consistent with this colloquial terminology (fresh-water chelonians elsewhere are generally referred to as turtles or terrapins) popular texts on the Chelidae in Australia have referred to these animals as tortoises (Worrell, 1970; Goode, 1967; Cann, 1978). I have followed this convention.

Characteristics of Eggs. A number of overlapping and synonymous terms have been coined by different authors in describing eggs. The terminology used to describe avian eggshells and shell membranes is extensive and, in some cases, confusing (Tyler, 1969). For descriptions of the eggshell and membranes in the eggs of *E. macquarii* I have followed M. Packard *et al.* (1982a) where possible. I used the terms soft-shelled and hard-shelled to describe the soft-shelled eggs of lizards, snakes and some chelonians which have been described as flexible-shelled (e.g. G. Packard *et al.*, 1981a), parchment-like (G. Packard *et al.*, 1977a), parchment-shelled (Ewert, 1979) and pliable-shelled (e.g. G. Packard

et al., 1979a) and the hard-shelled eggs of crocodilians and some lizards and chelonians which have been described as rigid-shelled (e.g. M. Packard *et al.*, 1982a), brittle-shelled (Ewert, 1979) and calcareous-shelled (G. Packard *et al.*, 1977a). Soft-shelled eggs are usually turgid throughout the incubation so the flexibility or pliability of the shell is not necessarily immediately obvious. Parchment-shelled implies a fibrous texture, yet many soft-shelled eggs have an external calcareous layer that obscures the underlying fibres. Hard-shelled eggs are usually rigid and brittle when laid, but this rigidity sometimes breaks down due to the cracking of the eggshell during incubation. Although all hard-shelled eggs are calcareous, many soft-shelled eggs have a significant calcareous component (M. Packard *et al.*, 1982a), which means that the terms calcareous-shelled and hard-shelled cannot be used interchangeably.

2.2 GENERAL METHODS

Source of Eggs. Most eggs were collected from nesting areas on the shores of Lake Bonney near Barmera and near Monteith on the River Murray in South Australia. A small number of eggs came from a third site near Milang on the shores of Lake Alexandrina (Fig. 2.1).

During the first two seasons (1978-79, 1979-80), eggs for field and laboratory studies were located or collected at Lake Bonney within 24 hours of oviposition. When located, the nests from which eggs were collected were assigned a number and carefully excavated. Each egg was also assigned a number, which was written on its upper surface, along with the nest number, with an HB grade graphite pencil. The egg was then removed from the nest and packed in sand from the nesting area in a plastic bucket or foam container and transported back to the laboratory by car.

Because of the high rate of predation on nests in the field, in following seasons it was necessary to collect gravid female tortoises from Lake Bonney prior to the nesting season to obtain enough eggs for the laboratory experiments. When eggs were required the female was injected with oxytocin (Pitocin R , Parke Davis Pty. Ltd., 32 Cawarra Rd, Caringbah, NSW) (1 $ml.kg^{-1}$) in the thigh to induce oviposition (Ewert & Legler, 1978). If no eggs were laid in two hours an additional half dose was given in the opposite thigh. This method proved to be more convenient than waiting for natural nesting because the actual time of nesting is impossible to predict with accuracy and the pattern of nesting varies from year to year depending on the weather conditions.

As each egg was laid it was assigned a number which was written on the side of the egg (between the poles) with an HB pencil. This number then represented the "top" of the egg. All eggs, both field collected

FIGURE 2.1 THE RIVER MURRAY IN SOUTH AUSTRALIA WITH THE THREE NESTING SITES EXAMINED MARKED:
1. LAKE BONNEY SITE;
2. MONTEITH SITE:
3. MILANG SITE.

and induced, were carefully maintained with the number uppermost throughout incubation.

In seasons where experimentation was based on eggs induced in the laboratory other eggs were also collected in the field where possible. These eggs were generally used in different experiments.

Handling of Eggs. All eggs were incubated in two litre plastic icecream containers that were covered with clear polythene plastic perforated with small holes so that water loss in the forced draft incubators was minimised but gas exchange was not precluded. Sand commonly used by nesting tortoises at Lake Bonney was collected for use as an incubation substrate in the laboratory. Sand from the same location was used in all experiments, it was sieved to exclude stones and wood and was heat sterilized before use. Only distilled water was used to moisten the substrate.

Statistics. Statistical analyses in this study followed Campbell (1974) and Zar (1974). In all cases means were given with ± one standard deviation and the sample size. Comparisons of means were made (e.g. using t-tests or ANOVA) after the variances were shown to be equal using the F distribution. Linear regressions were calculated by the method of least squares and the coefficient of determination (r^2) given as an indication of goodness of fit. For non-linear equations the coefficient of determination refers to the log or semi-log transformed data. Statistical significance was accepted where p-values were less than 0.05.

3

PHYSICAL CHARACTERISTICS OF EGGS

3.1 INTRODUCTION

Eggs of the Chelonia range from hard-shelled to soft-shelled (G. Packard *et al.*, 1977a), with many intermediates (Ewert, 1979). Soft-shelled eggs, such as those of *Caretta caretta*, have an outer calcareous layer composed of a loosely organised and open matrix with poorly defined individual shell units and pores. Hard-shelled eggs, like those of *Kinosternon bauri*, have a relatively thick calcareous layer composed of tightly fitting, discrete shell units with well defined pores (M. Packard *et al.*, 1982a) where four or more shell units meet (Tullett, 1975). Beneath the shell lie the shell membranes (G. Packard *et al.*, 1977a). A few species with hard shells (e.g. *Testudo graeca*, Young, 1950) have a thin cuticle on the outside of the shell.

The structure of the eggshell and membranes regulates many of the physiological functions of the egg. For example, soft-shelled eggs swell as they imbibe water; some species produce larger, more successful hatchlings if their eggs are incubated in moist conditions (G. Packard *et al.*, 1980, 1981a,b). On the other hand, a rigid eggshell precludes the uptake of water (M. Packard *et al.*, 1982a) and the hatching success and size of hatchlings are independent of the hydric conditions during incubation (G. Packard *et al.*, 1981c). The rate of diffusion of gases through the shell is also determined by its structure (Wangensteen *et al.*, 1970/71; Ar *et al.*, 1974).

As part of this investigation, structure of the eggshell and membranes of *E. macquarii* were examined so that -

(a) relationships between structure and response to the hydric conditions during incubation (Chapter 6) could be compared with data for other species (M. Packard *et al.*, 1982a), and

(b) factors limiting diffusion through the shell could be identified (Chapter 5).

Because of differences in the structure of avian and chelonian eggs, M. Packard and Packard (1979) suggested the use of simple terminology to describe chelonian eggshells, thereby avoiding the extensive terminology of avian eggs (Tyler, 1969). For convenience I use the term *eggshell* to mean the calcareous part of the shell (or true shell (Tyler, 1969)) and *shell membranes* to describe the complex of fibre layers underlying, but in intimate contact with, the shell.

The process of shell formation is assumed to be essentially the same in the Chelonia as it is in birds, because of similarities in the structure of the eggshell of *Trionyx spiniferus* and birds (M. Packard & Packard, 1979). However, unlike birds there is no regionalisation of the oviduct in chelonians, the shell membranes, organic cores and shell all being deposited in the shell-forming part of the oviduct; this implies different processes of shell formation in chelonians and birds (Solomon & Baird, 1977). Similarities between the structure of the eggshell and membranes of *E. macquarii* and those of other species would imply the same process of egg formation in different species of chelonians.

The shape of the eggs of *E. macquarii* is described and compared to the number of eggs per clutch. Chelonians which lay small clutches (fewer than 10 eggs) usually have elongate (ellipsoid) eggs, whereas large clutches usually contain spherical eggs. This may be an adaptation that enables more eggs to be packed into the oviducts (Ewert, 1979). Exceptions (where egg shape follows taxonomic lines) include the Kinosternidae and Emydidae, with elongate eggs, and the Trionychidae, with spherical eggs. Australian Chelidae have elongate eggs, whereas Neotropical species

have spherical eggs (Ewert, 1979).

The length, breadth and elongation of the eggs of *E. macquarii* are compared to each other to establish the more important parameter, shape or volume. The length and breadth of elongate eggs have been positively correlated in some chelonians (e.g. *Malaclemys terrapin*, Montevecchi & Burger, 1975) but not others (e.g. *Chrysemys picta* and *Terrapene carolina*, Tucker *et al.*, 1978). A positive correlation indicates that egg shape is conserved within the normal range of egg sizes, and a negative correlation indicates that volume is conserved (Preston, 1969). There is little intraspecific variation in the elongation (length x breadth^{-1} (Preston, 1968)), for species in which egg shape is conserved (e.g. birds, Preston, 1969).

As female chelonians become larger they may -

(1) maintain their clutch size,

(2) lay more eggs of the same size in a clutch,

(3) lay the same number of larger eggs, or

(4) increase the number and size of eggs.

Although larger eggs are produced by larger *Chelydra serpentina* (Yntema, 1970), there is no such relationship in *Malaclemys terrapin* (Montevecchi & Burger, 1975). The number of eggs in a clutch and the size of the mother are positively correlated in many species (e.g. *Pseudemys scripta*, Cagle, 1944; Gibbons, 1982; *Kinosternon bauri*, Einem, 1956; *Sternotherus odoratus*, Tinkle, 1961; *Clemmys guttata*, Ernst, 1970; *Chelonia mydas*, Bustard, 1972; *Chelydra serpentina*, Yntema, 1970; White & Murphy, 1973; *M. terrapin*, Montevecchi & Burger, 1975; *Sternotherus minor*, Cox & Marion, 1978; *Podocnemis expansa*, Alho & Pādua, 1982). Positive correlations have been demonstrated for *Chrysemys picta* over its whole geographic range, but comparisons within single populations indicate no correlation (Cagle, 1954;

Gibbons & Tinkle, 1969; Tucker *et al.*, 1978). This led Gibbons and Tinkle (1969) to conclude that the two parameters are independently influenced within particular populations. The correlation between body size and clutch size varies between localities in *Sternotherus odoratus* also (Tinkle, 1961). In three populations of *Geochelone gigantea* the number of eggs per clutch is related to population density (Swingland & Coe, 1979). Variation in the number of eggs per clutch in an individual from year to year is as great as the variation in the population in one year in *Kinosternon subrubrum* and *Deirochelys reticularia*, but not in *P. scripta* (Gibbons, 1982). There is no correlation between the number of eggs per clutch and body size in *Terrapene carolina* but larger specimens do lay larger eggs (Tucker *et al.*, 1978). Therefore, in general, there is an increase in the mass of clutches as the female grows resulting from either an increase in the number of eggs in a clutch (e.g. *M. terrapin*) or an increase in egg size (e.g. *T. carolina*).

Interspecific studies on chelonians (Iverson, 1977) and lizards (Vitt, 1977) have shown positive correlations between the number of eggs per clutch and female size, but with a great deal of intraspecific variation.

The comparison of egg and hatchling sizes made in *E. macquarii* was related to changes in egg size with female size to see whether the fitness of hatchlings produced by one female may change throughout its reproductive life. In the Chelonia large eggs generally give rise to large hatchlings (Yntema, 1968, 1970; Tucker *et al.*, 1978; Cox & Marion, 1978; Swingland & Coe, 1979). Correlations between egg size and hatchling size are particularly important in discussions of the evolution of egg size because it is generally assumed that larger hatchlings have a better chance of survival than smaller ones (M. Packard *et al.*, 1982a). Tucker

et al. (1978) summarized the ways in which larger size may be advantageous; larger hatchlings may -

(1) have a better chance of avoiding predators,

(2) be better able to dig out of the nest chamber,

(3) be able to store more yolk,

(4) be more mobile,

(5) be able to subdue larger and more varied prey items.

The thickness of the shell from fresh and hatched eggs was measured in *E. macquarii* to establish the extent of shell thinning due to resorption of calcium during development and to examine variations in shell thickness in different regions of the shell. The eggshell of domestic chickens is generally thicker at the narrow pole, becoming thinner towards the blunt end (Olsson, 1936), but there are often variations in this trend. However the thickness of the shell is consistent in one plane through the egg (Tyler, 1961a, Tyler & Geak, 1965). Variations in thickness can be correlated with the strength requirements of the egg (Tyler, 1969). Except during oviposition the stresses on the shell of chelonian eggs are minimal, and in soft-shelled eggs the calcareous structure, which provides much of the strength, is reduced (M. Packard *et al.*, 1982a). Some calcareous component in the shell is necessary because it is the source of calcium for ossification of embryonic bones (Bustard *et al.*, 1969). Calcium is dissolved from the shell by the embryo during development (M. Packard & Packard, 1979).

3.2 MATERIALS AND METHODS

3.2.1 Linear Dimensions and Mass of Eggs and Tortoises

Gravid female tortoises, collected in Lake Bonney prior to the nesting season, were weighed and measured, as described in Chapter 8, before being injected in the thigh with oxytocin (Chapter 2). These tortoises were retained in fibreglass holding tanks and fed with varying amounts of mosquitofish, *Gambusia affinis*, prawns, *Paratya australiensis*, yabbies, *Cherax destructor*, carp, *Cyprinus carpio* (all from the River Torrens) and sheep liver.

Egg lengths and breadths were measured to 0.01 mm with a dial vernier caliper. Eggs from eleven field clutches and twenty induced clutches and part clutches were measured. All eggs were weighed to the nearest milligram, except those in the 1980-81 season which were weighed to the nearest 10 mg. Field eggs (20 clutches and part clutches) were weighed on return to the laboratory, usually within 48 hours of being laid. Induced eggs (31 clutches and part clutches) were weighed as they were laid.

3.2.2 Mass of Fresh Egg : Hatchling Relationship

In the 1981-82 trials each egg was separated by plastic mesh from others so that the hatchling from each egg could be identified. As hatchling mass is independent of the water potential of the incubation substrate (Chapter 6), a comparison was made of hatchling mass to fresh egg mass. Treatments included eggs incubated at (i) 30°C (121), (ii) 25°C (9) and (iii) 20°C for part of the incubation and then transferred to 30°C (3). A single factor ANOVA was used to determine the differences between treatments.

3.2.3 Water Content of Fresh Eggs, Embryos and Hatchlings

The water content of fresh eggs was determined in two ways.

1. Eggs were cut open with an edge-cutting disc in a dental drill and the yolk, albumen and shell separated, weighed and placed in tared containers over silica gel, dried to constant mass under a vacuum at room temperature and the percentage water content calculated. All eggs were cut open within a week, and most within 48 hours of being laid.
2. Whole eggs were placed over silica gel at 25°C, at atmospheric pressure, and dried to constant mass. Eggs used in this analysis were a series placed in desiccators on days 1, 15, 30, 45 and 57 of incubation (Chapter 6).

Fresh egg mass, final dry mass and percent water content were each compared between trials using a single factor ANOVA.

Embryos of different ages were removed from eggs during the incubation, weighed, preserved in 70% ethanol, dried to constant weight at 60°C and their water content calculated.

Hatchlings were brushed free of adhering sand and weighed as soon as possible after hatching (within 8 hours), killed by freezing and then dried over silica gel at room temperature under vacuum. The wet mass, dry mass and percent water content of hatchlings incubated under different conditions of water potential were compared using a single factor ANOVA.

3.2.4 Volume and Surface Area of Eggs

Volumes of eggs were determined by displacement of isopropyl alcohol (Hoyt, 1976) in a glass vessel 35 mm in diameter. The mean of four

measurements on each egg was calculated. Surface areas of 131 eggs from 21 clutches were calculated by the equation derived by Hoyt (1976), modified for the mean elongation of the eggs of *E. macquarii*:

$$\text{Surface area} = 5.039\ \text{Volume}^{2/3} \qquad (3.1)$$

3.2.5 Thickness of Eggshell and Shell Membranes

The thickness of the shell of all eggs that hatched in the "water relations experiment" (Sections 6.3.2, 6.4.2) and most of the eggs in the "pyramid experiment" (Sections 6.3.3, 6.4.7) was measured. Shell membranes were peeled from the portions of shell to be measured. Some shells were soaked in 2% NaOH overnight to free them from the membranes. A screw micrometer was modified by having a ball-bearing fixed to it with sealing wax. The inner surface of the eggshell was placed on the ball bearing and the shell thickness measured to 0.001 mm. Because the thickness of the shell varied in different places on the egg the mean of five measurements on fragments from the equatorial area and five from the end (pole) of the egg were calculated.

Examination of the variation in shell thickness from different regions of the egg was possible on egg 4607 in which the shell came away from the membrane intact. This egg had been cut in halves along the equatorial plane with an edge-cutting disc in a dental drill early in development. Forty-two measurements of eggshell thickness were made at approximately 2 mm intervals around the long axis of the egg and twelve around the equatorial plane. Most other eggshells fragmented and comparative measurements could not be made.

The thickness of shell from the equator and poles of eight fresh

eggs from four clutches was measured with the membranes intact. They were then soaked in 10% Na_2S at room temperature for 48 hours to remove the membranes. The shell thickness was then measured and the thickness of the membranes calculated by subtraction. The mean of five measurements was used.

3.2.6 Fine Structure of Eggshell and Membranes

Scanning electron microscopy (ETEC Autoscan) was used to examine the fine structure of the eggshell. Eggshell fragments were boiled in 2% NaOH (by weight) for ten minutes to remove organic matter (Tyler & Geake, 1953a), rinsed in distilled water and dried under vacuum over silica gel at room temperature. The procedure was repeated if any organic matter was shown to remain on examination under a binocular dissecting microscope. Casts of some specimens were made in Spurr's resin (Tompa, 1980). The eggshell was dissolved from the cast with concentrated HCl; this left a jelly-like organic material, probably the organic matrix of the shell (Tyler, 1969), which was then washed from the cast with a stream of water from a Pasteur pipette.

Shell membranes were prepared for SEM by dissolving the inorganic fraction in 2% HCl. Membranes were torn with a tangential force to separate the layers of fibres.

Specimens were glued to stubs using DAG®915 (Silver in M.I.B.K., Acheson Colloids Co., Prince Rock, Plymouth) and vacuum coated with 200 Å of gold/palladium. An accelerating voltage of 20 kV was used and micrographs were taken on Kodak Panatomic X120 film.

Measurements were made from the micrographs to 0.01 mm with a dial

vernier caliper. Variations up to 6% were measured on the same structure in micrographs at two different magnifications so statistical comparisons with measurements made by different methods (e.g. screw micrometer) were not made.

Shell samples from thirteen eggs that had been treated to remove the organic fraction were viewed under a binocular dissecting microscope. Pores were assumed to transmit light through the shell. The number of pores within a 6.25 mm^2 grid were counted. Five replicates from the middle and five from the end of each egg were made at random and the mean number of pores per mm^2 calculated for these two regions of the shell.

3.3 RESULTS

3.3.1 Linear Dimensions and Mass of Eggs

Mean length, breadth and mass are calculated for all eggs measured (Table 3.1). Only fresh egg masses (i.e. within 48 hours of oviposition) are used. The mean breadth is plotted against mean length (Fig. 3.1) and mean mass (Fig. 3.2) for each clutch or part clutch measured. Mean length is also plotted against mean mass (Fig. 3.3). There are significant positive correlations between length (L) and breadth (B), length and mass (M) and breadth and mass. Semilog transformed regressions fit the data better than linear regressions:

$$B = 8.961 \ln L - 10.124 \quad (n = 31, r^2 = 0.278, 0.001<p<0.0025) \tag{3.2}$$

$$B = 7.067 \ln M + 5.468 \quad (n = 31, r^2 = 0.833, p<<0.0005) \tag{3.3}$$

$$\ln L = 0.0374 M + 3.192 \quad (n = 31, r^2 = 0.669, p<<0.0005) \tag{3.4}.$$

TABLE 3.1 MEAN VALUES AND RANGES FOR CHARACTERISTICS OF THE EGGS OF *E. MACQUARII*

	$\bar{x}$	S.D.	Range	n
Length (mm)	36.11	2.17	30.54 - 46.90	523
Breadth (mm)	22.02	1.28	18.92 - 24.01	523
Weight (g)	10.423	1.246	6.75 - 14.34	871

Elongation, calculated using the mean values for each clutch, is relatively constant (range 1.50 - 1.74 excluding one clutch of unusually

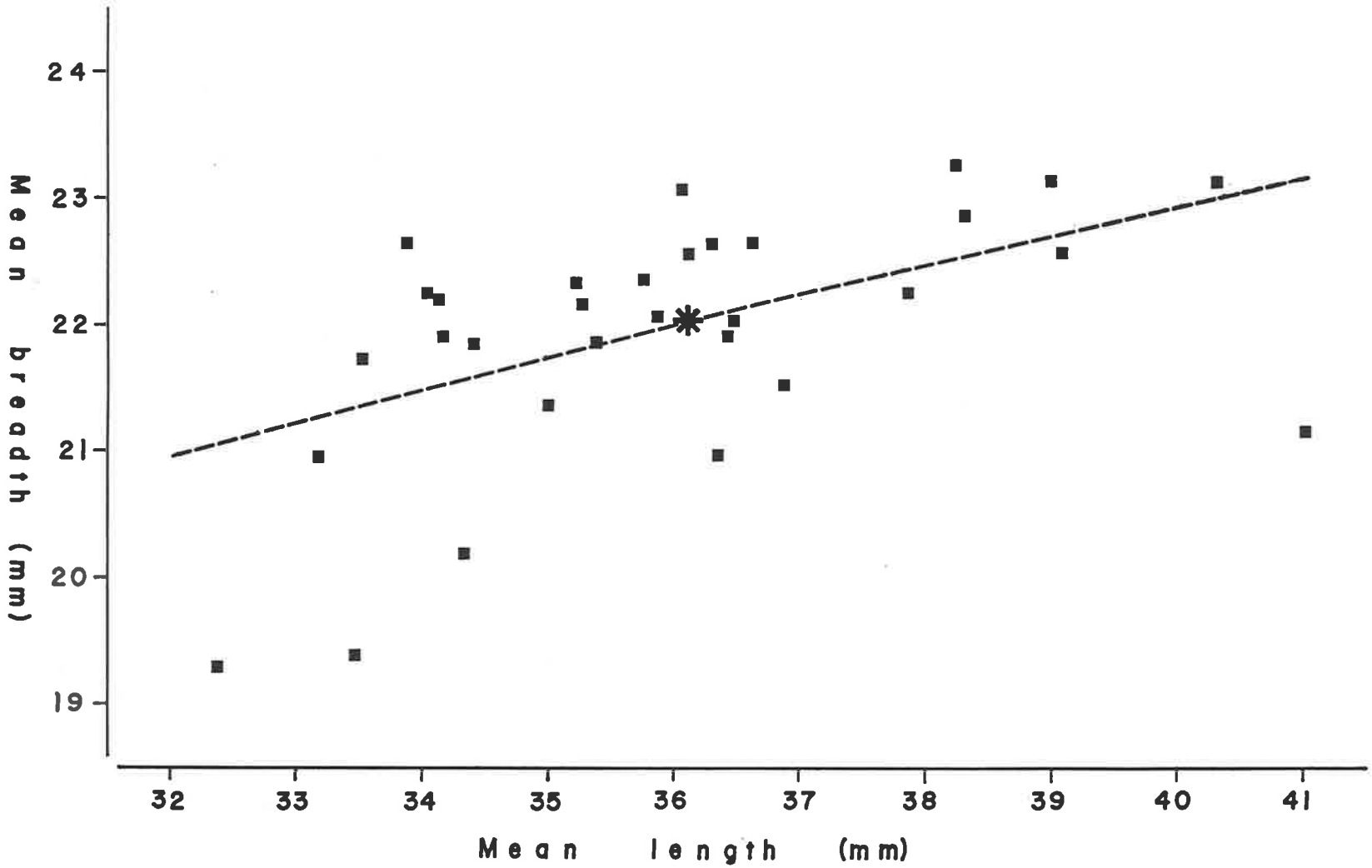


FIGURE 3.1 EGG BREADTH (mm) PLOTTED AGAINST EGG LENGTH (mm). EACH VALUE REPRESENTS THE MEAN FOR ALL EGGS FROM A CLUTCH OR PART CLUTCH (n = 31). ✱ = OVERALL MEAN. REGRESSION LINE (EQUATION 3.2) IS FITTED.

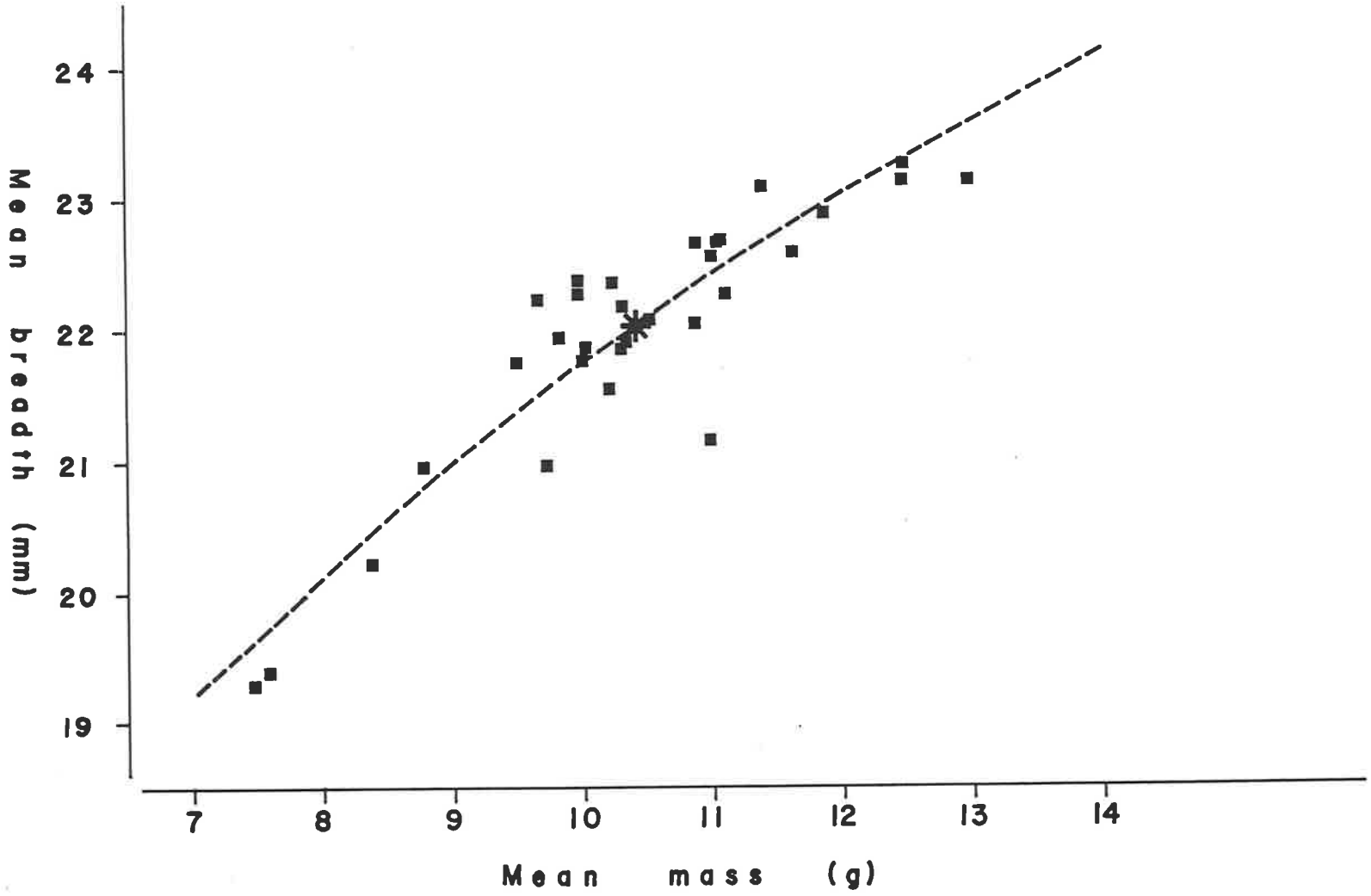


FIGURE 3.2 EGG BREADTH (mm) PLOTTED AGAINST EGG MASS (g). EACH VALUE REPRESENTS THE MEAN FOR ALL EGGS FROM A CLUTCH OR PART CLUTCH (n = 31). ✱ = OVERALL MEAN VALUE. REGRESSION LINE (EQUATION 3.3) IS FITTED.

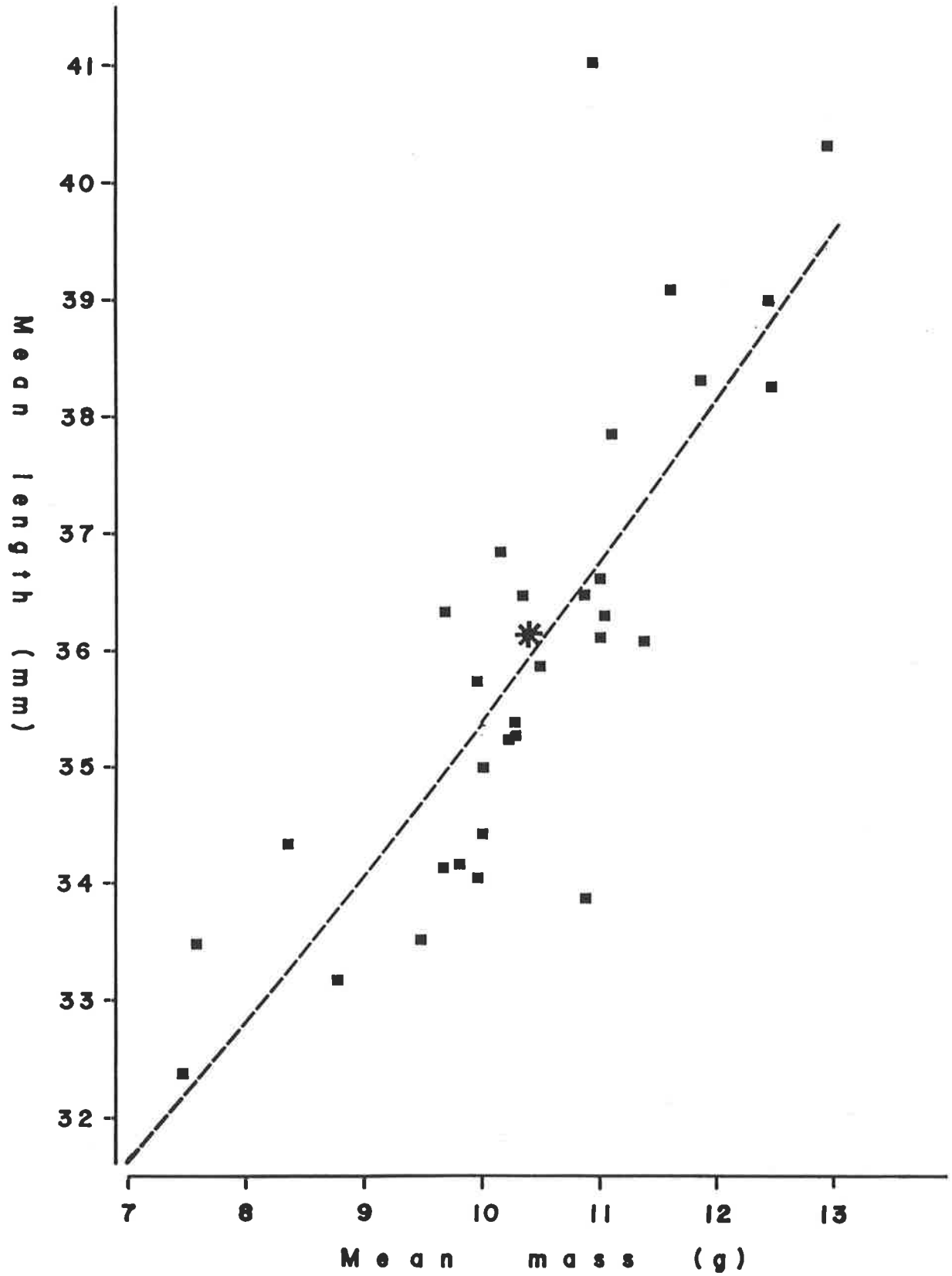


FIGURE 3.3

EGG LENGTH (mm) PLOTTED AGAINST EGG MASS (mm). EACH VALUE REPRESENTS THE MEAN FOR ALL EGGS FROM A CLUTCH OR PART CLUTCH (n = 31). ✱ = OVERALL MEAN VALUE. REGRESSION LINE (EQUATION 3.4) IS FITTED.

long eggs) over the range of egg breadths (Fig. 3.4). Mean elongation is 1.64 ± 0.09 (n = 31). The slope of the linear regression comparing elongation to egg breadth is not significantly different from zero ($0.05<p<0.10$) which indicates that egg shape is conserved over the range of egg sizes.

3.3.2 Female Tortoise : Egg Relationship

The method of obtaining eggs from *E. macquarii* with oxytocin is not often 100% successful so comparisons between the number of eggs in a clutch and the size of the female are not possible. However, mean masses (g) of the eggs obtained from each female are plotted against the masses of the females less the egg mass (g) (Fig. 3.5). A linear regression relating the natural log of mean egg mass (lnM) against tortoise mass (T, g) fits the data better than the regression relating mean egg mass to tortoise mass ($r^2 = 0.413$, n = 32 compared to $r^2 = 0.434$, n = 32). The regression is:

$$\ln M = (2.1915 \times 10^{-4})\, T + 1.72 \qquad (3.5).$$

Plastron length was used as an index of size (Chapter 8). A regression relating the natural log of mean egg mass (lnM) to curved plastron length (P) (Fig. 3.6) is a better fit than that relating mean egg mass to plastron length ($r^2 = 0.392$, n = 30 compared to $r^2 = 0.445$, n = 30). The calculated regression is:

$$\ln M = 0.0076\, P + 0.465 \qquad (3.6).$$

Variances of the mean fresh masses and sizes of wild collected and induced eggs (Table 3.2) are statistically equal (variance ratio distribution (F) (Campbell, 1974)). The mean fresh mass of eggs collected in the field is not significantly different ($t = 1.114$, d.f. = 49, $0.20<p<$

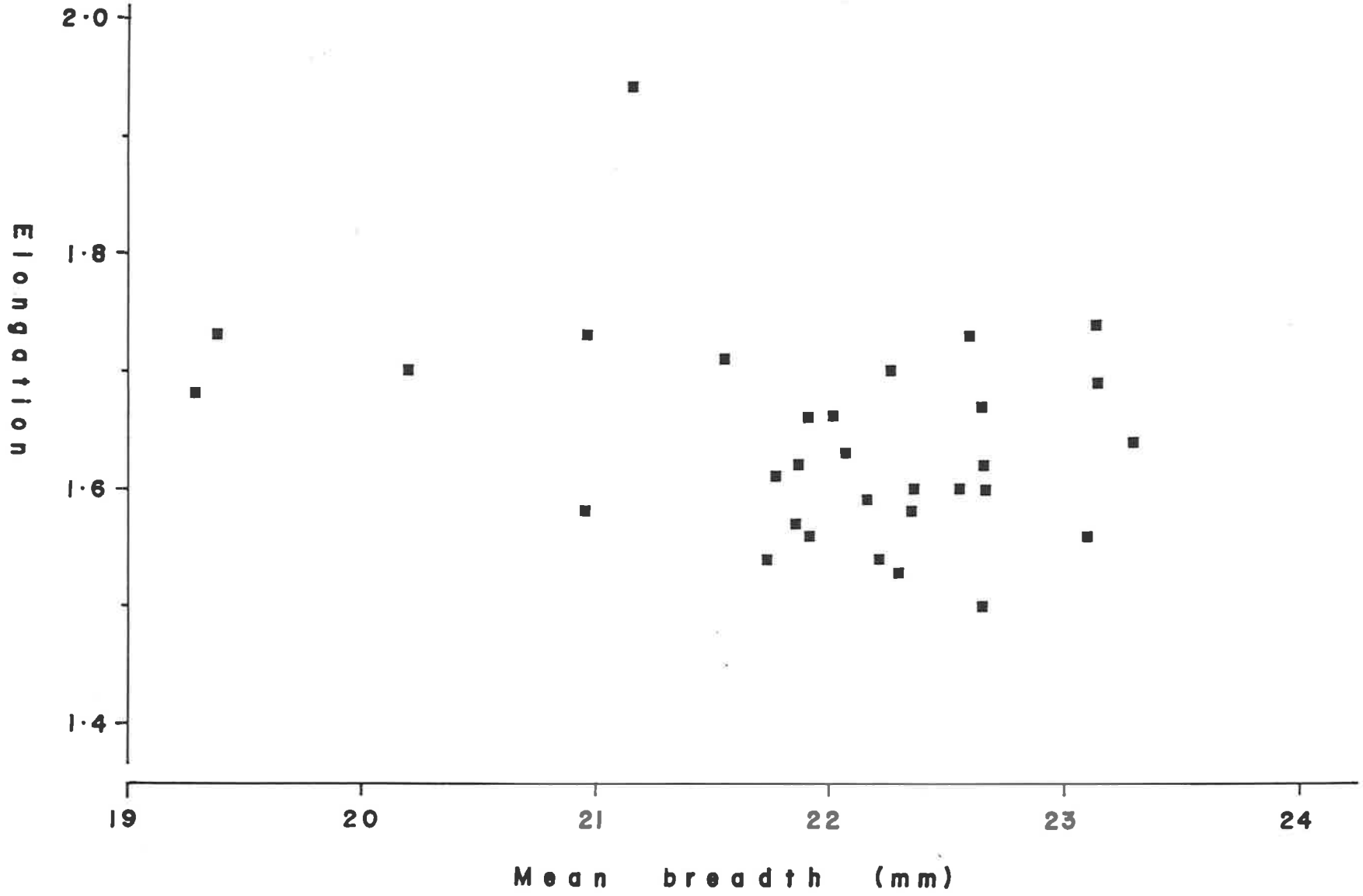


FIGURE 3.4 ELONGATION CALCULATED FROM THE MEAN LINEAR DIMENSIONS OF EGGS IN EACH CLUTCH PLOTTED AGAINST THE MEAN BREADTH OF THE EGGS IN THE CLUTCH (n = 31).

FIGURE 3.5

Mean egg mass for each clutch induced from tortoises in the laboratory plotted against the mass of the tortoise, less the weight of the eggs collected.
Regression line (equation 3.5) is fitted to the data.
Numbers in parentheses indicate the number of eggs in the clutch.

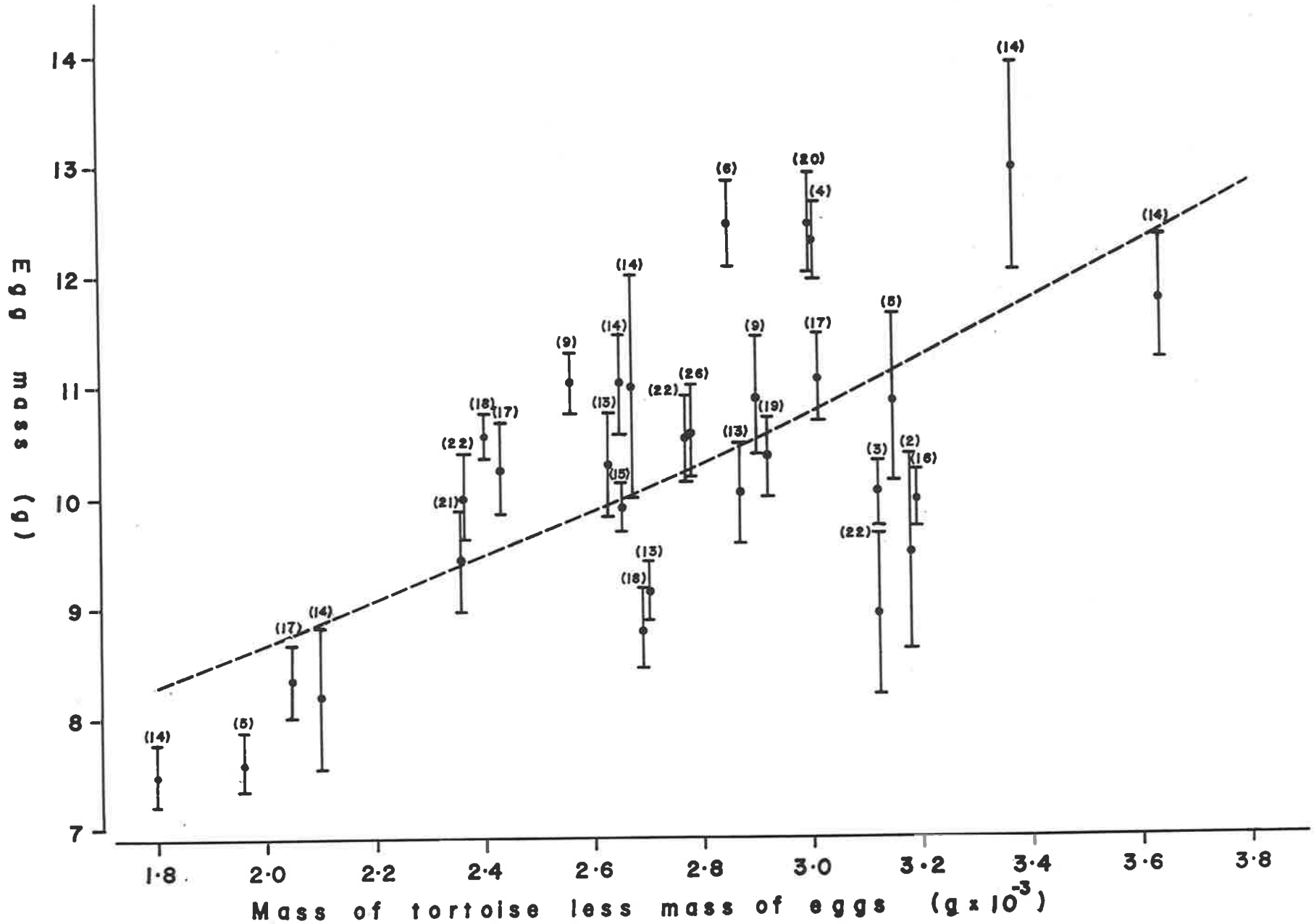
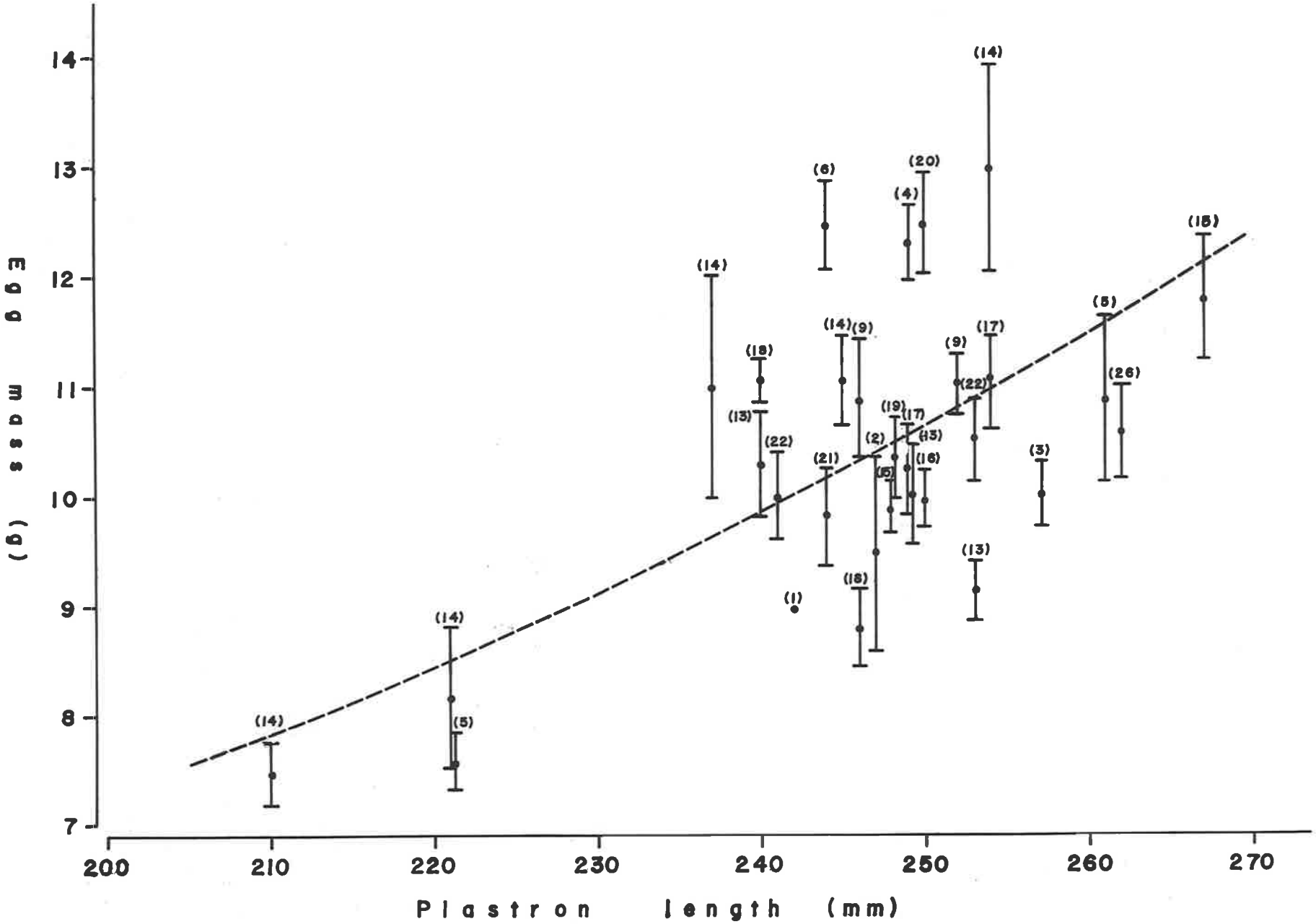

Egg mass (g)
14
13
12
11
10
9
8
7
1·8
2·0
2·2
2·4
2·6
2·8
3·0
3·2
3·4
3·6
3·8
Mass of tortoise less mass of eggs (g x 10⁻³)

FIGURE 3.6

Mean mass of eggs for each clutch induced from tortoises in the laboratory plotted against the curved plastron length of the tortoise.
Regression line (equation 3.6) is fitted to the data.
Numbers in parentheses indicate the number of eggs in the clutch.


Egg mass (g)
14
13
12
11
10
9
8
7
200
210
220
230
240
250
260
270
Plastron length (mm)
(14)
(6)
(20)
(4)
(14)
(15)
(5)
(14)
(9)
(17)
(18)
(9)
(26)
(22)
(19)
(17)
(13)
(2)
(15)
(22)
(21)
(16)
(3)
(5)
(13)
(1)
(18)
(14)
(5)

0.30) from those induced in the laboratory. However, the clutch size differs significantly ($t = 4.696$, d.f. = 49, $p<<0.001$) as a result of many of the females not laying all their eggs when induced with oxytocin.

TABLE 3.2

Category	n	Egg Mass (g)		Number of Eggs	
		$\bar{x}$	S.D.	$\bar{x}$	S.D.
Field Nests	19	10.627	1.048	21.5	5.2
Induced Clutches	32	10.221	1.365	13.4	6.3
Significance		NS		***	

Characteristics of clutches collected in the field and obtained by induction with oxytocin in the laboratory. Field collected and induced eggs compared using t-test. See text for probabilities.

Mean egg breadth (B, mm) and mean egg length (L, mm) are compared to the plastron length (P, mm) of the tortoises from which the eggs came (Figs. 3.7, 3.8). Linear regressions give:

$$B = 0.067\ P + 5.566 \quad (n = 24,\ r^2 = 0.626,\ p<<0.001) \qquad (3.7)$$

and

$$L = 0.058\ P + 21.285 \quad (n = 24,\ r^2 = 0.104,\ p>0.05) \qquad (3.8)$$

which indicates that egg breadth is positively correlated to the size of the female tortoise and egg length is not.

3.3.3 Mass of Fresh Egg : Hatchling Relationship

The masses of hatchlings incubated at the three different temperatures are pooled because a single factor ANOVA shows no significant difference between treatments ($F = 1.554$, d.f. = 2, 130, $0.10<p<0.25$). The

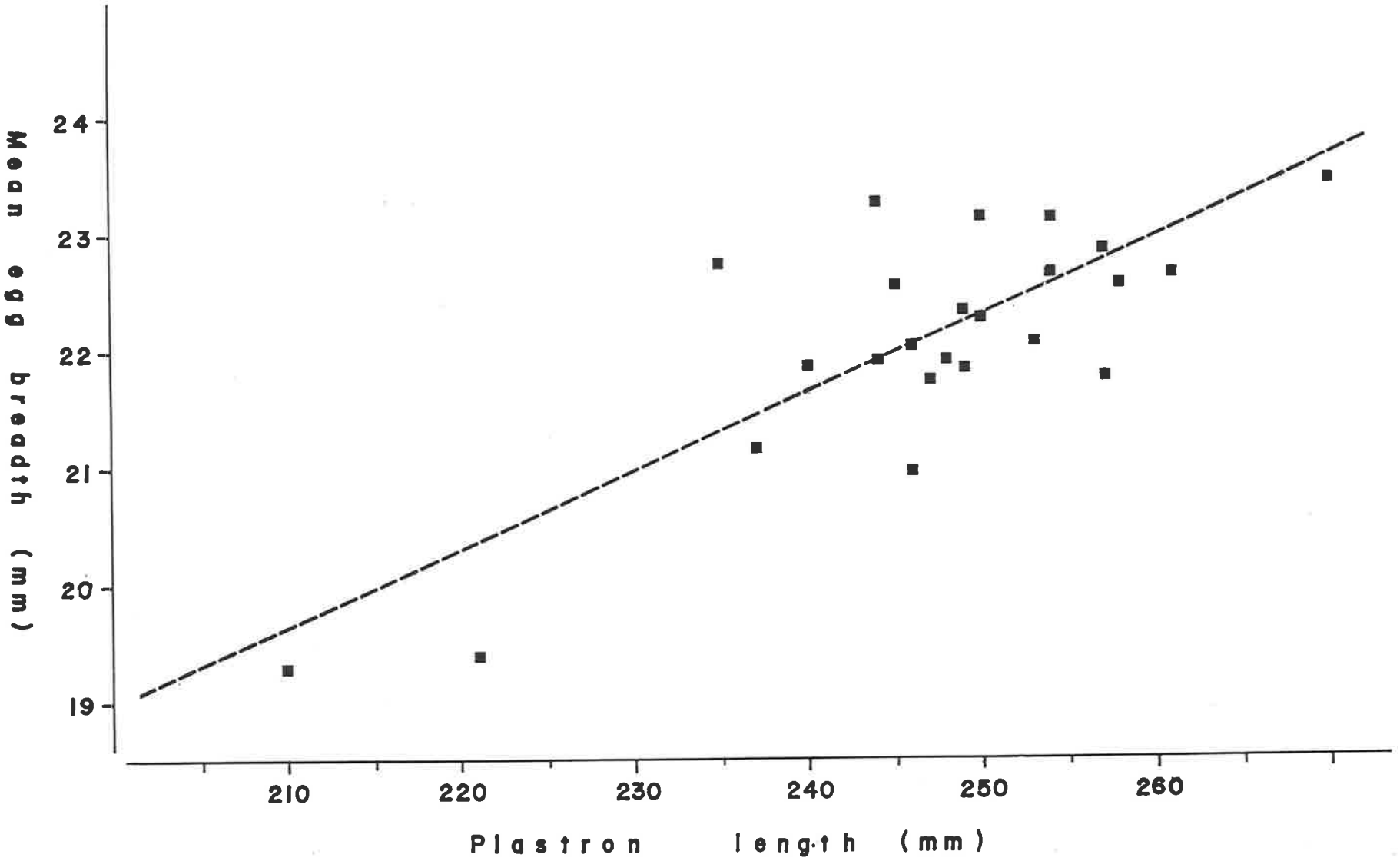


FIGURE 3.7 EGG BREADTH (mm) PLOTTED AGAINST PLASTRON LENGTH (CURVED) (mm) OF THE MOTHER (n =24). REGRESSION LINE (EQUATION 3.7) IS FITTED.

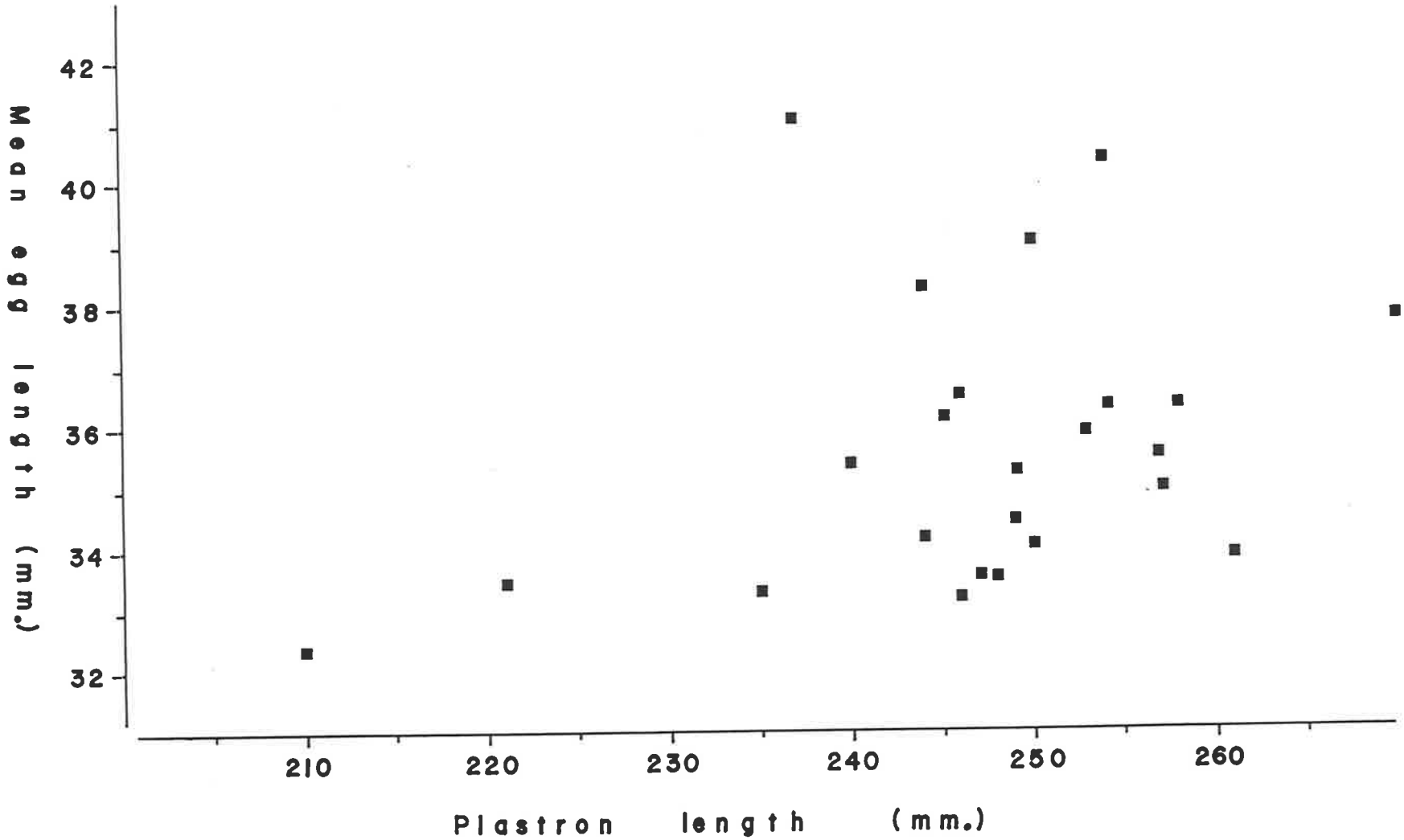


FIGURE 3.8 EGG LENGTH (mm) PLOTTED AGAINST PLASTRON LENGTH (CURVED) (mm) OF THE MOTHER (n = 24).

hatchling mass of 5.093 (± 0.458) g is 48.06% (± 3.25%) of the fresh egg mass (n = 133). The linear regression of hatchling mass (H) against the natural log of fresh egg mass (Fig. 3.9) is:

$$H = 3.331 \ln M - 2.766 \quad (n = 133, r^2 = 0.604) \qquad (3.9).$$

3.3.4 Water Content of Fresh Eggs, Embryos and Hatchlings

When yolk, albumen and shell of 113 eggs were weighed separately (Table 3.4) a mean 0.193 g of material (1.96%) was lost: there was shell lost when cut with the edge-cutting disc, and some spillage and evaporative loss of fluid. The percentage of yolk may be slightly underestimated and that of albumen overestimated because it is difficult to separate the components of the egg without rupturing the yolk membrane once the embryo has adhered to the inner shell membrane. However, comparisons with unbroken yolks indicated no serious biases. The fractions of 16 eggs were dried and their water contents calculated (Table 3.5).

TABLE 3.4 MEAN WEIGHT (g), STANDARD DEVIATION AND COMPONENT FRACTIONS FOR 113 EGGS OF *E. MACQUARII*

Fraction	Weight (g)	S.D.	Percentage
Fresh Egg	9.815	1.384	
Yolk	3.655	0.043	37.24
Albumen	4.602	0.709	46.89
Shell	1.365	0.283	13.91
Total	9.622	1.333	98.04
Loss	0.193		1.96

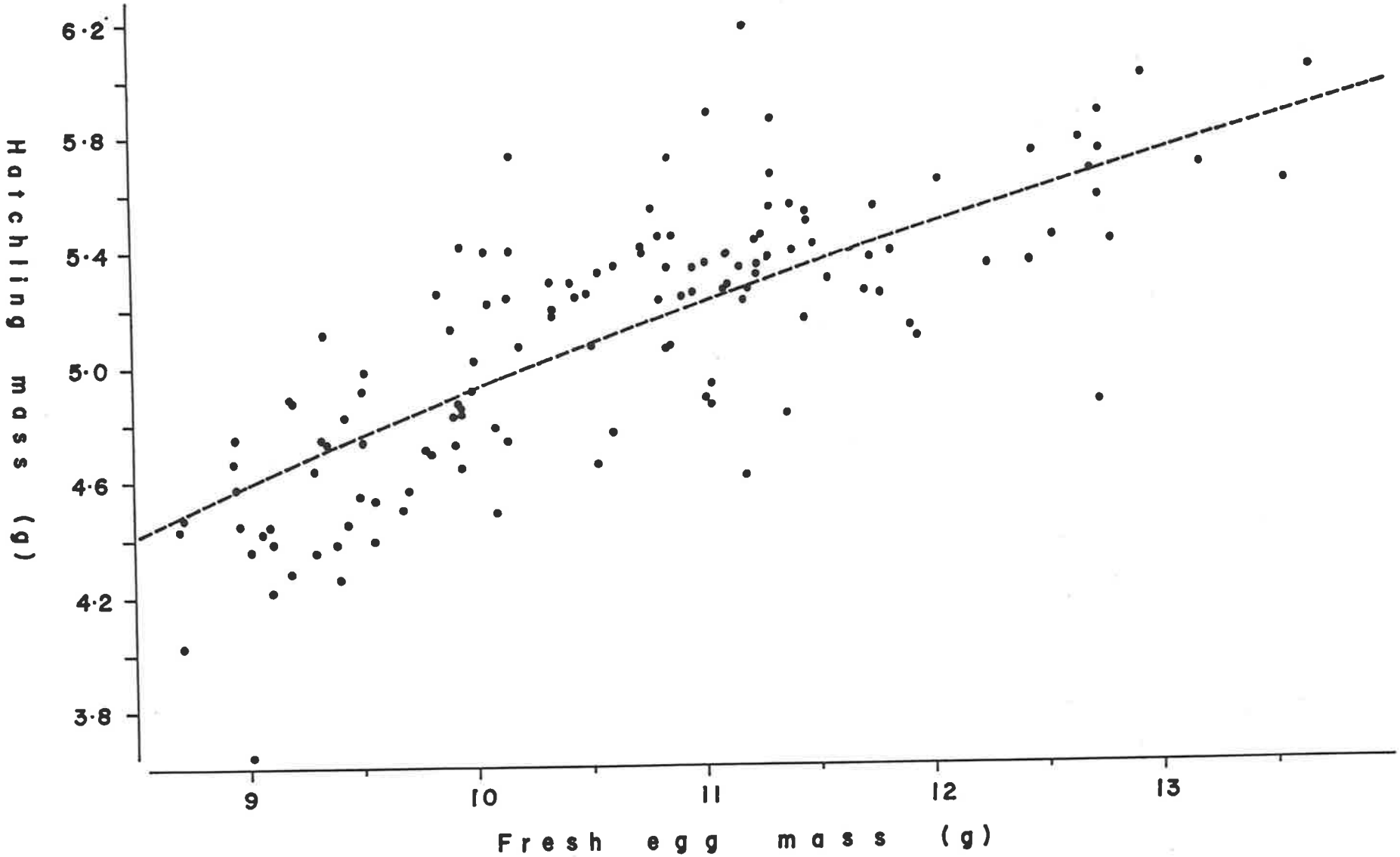


FIGURE 3.9 HATCHLING MASS (g) AGAINST FRESH EGG MASS (g). REGRESSION LINE (EQUATION 3.9) IS FITTED (n = 133).

TABLE 3.5 MEAN WET AND DRY WEIGHTS (g) AND PERCENT WATER CONTENT OF COMPONENT FRACTIONS OF 16 EGGS

Fraction	Wet Weight	Dry Weight	Percent Water
Yolk	4.549	1.459	67.93
Albumen	5.055	0.233	95.39
Shell	1.588	1.233	22.29
Total	11.192	2.926	73.86

The fresh and dry mass of eggs desiccated at different times during the incubation are statistically equal (ANOVA) so the results are pooled. The eggs of *E. macquarii* contain 76.12% water (n = 27, Table 3.6). The fresh mass, dry mass and water contents (percent) of hatchlings incubated in substrates of different water potential are not significantly different (ANOVA) (n = 110) also, so the results are pooled. The fractional water content of whole eggs is similar to that of hatchlings (Tables 3.5, 3.6).

TABLE 3.6 THE FRESH (WET) AND DRY MASS (g) AND PERCENT WATER CONTENTS OF EGGS AND HATCHLINGS

Category	Fresh (wet) Mass (g)		Dry Mass (g)		Percent Water		n
	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Whole Egg	10.666	1.112	2.549	0.314	76.12	1.32	27
Hatchling	5.074	0.474	1.170	0.152	76.95	1.76	110

In the early stages of incubation 95% of the yolk-free embryo is water. Halfway through incubation this begins to fall to 77% at hatching (Fig. 3.10).

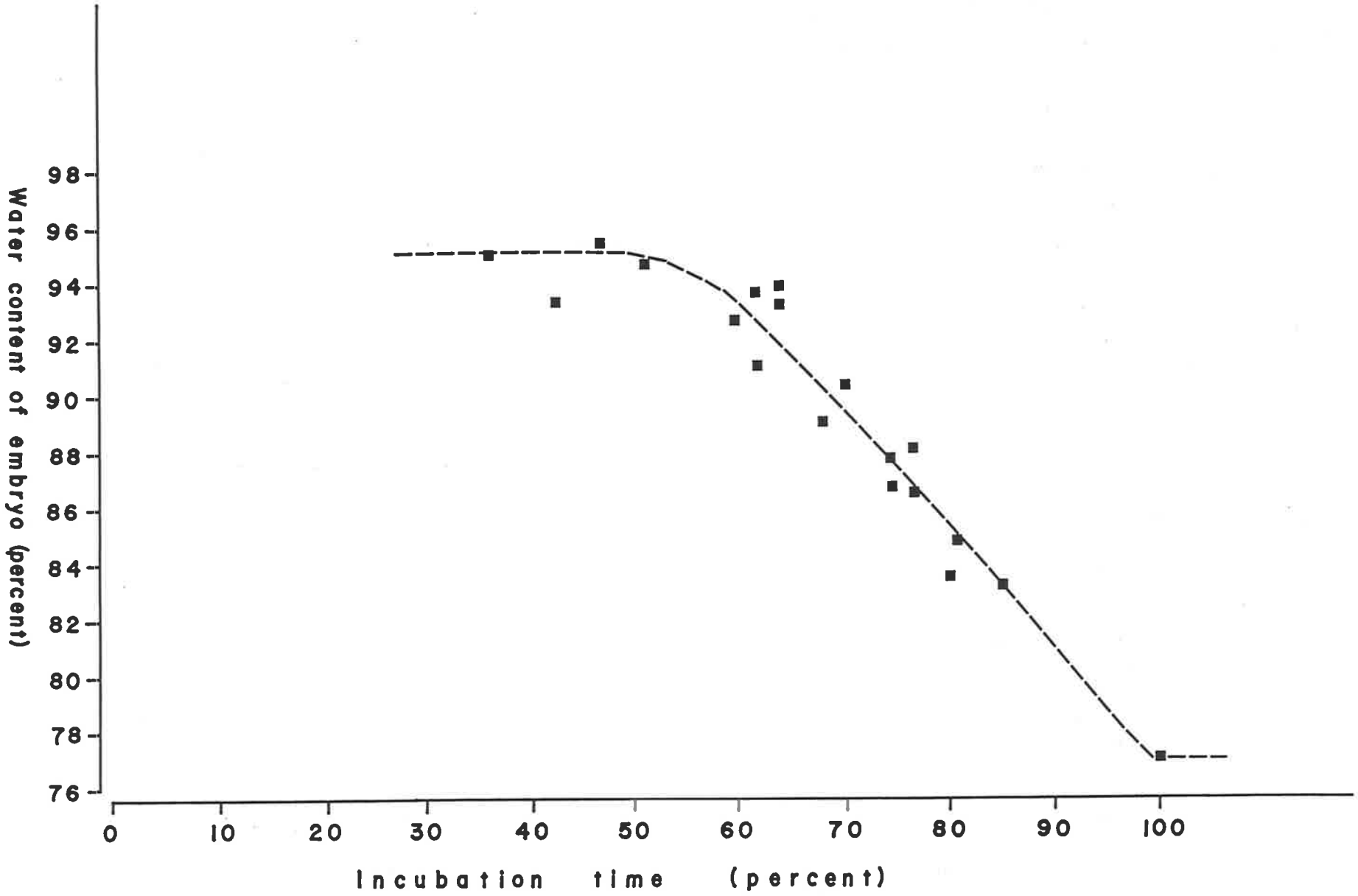


FIGURE 3.10 THE WATER CONTENT OF EMBRYOS AND EXTRA-EMBRYONIC MEMBRANES THROUGHOUT THE INCUBATION PERIOD FOR 18 EMBRYOS INCUBATED AT 30°C. HATCHLING EMBRYONIC WEIGHT IS MEAN OF 110 MEASUREMENTS FROM TABLE 3.6. LINE FITTED BY EYE.

3.3.5 Volume and Surface Area of Eggs

The fresh mass and linear dimensions of eggs for which volume was measured and surface area was calculated were slightly larger than "average" sized eggs (Tables 3.1, 3.7).

TABLE 3.7 MEAN AND STANDARD DEVIATION OF FRESH MASS (g), LINEAR DIMENSIONS (mm), VOLUME (cm^3) AND SURFACE AREA (cm^2) OF 131 EGGS FROM 21 CLUTCHES

Measurement		Mean	S.D.
Fresh mass	(g)	10.78	1.11
Length	(mm)	35.62	1.70
Breadth	(mm)	22.48	0.90
Volume	(cm^3)	9.686	1.021
Surface area	(cm^2)	22.869	1.629

3.3.6 Thickness of Eggshell and Membranes

The equatorial shell from both hatched and fresh eggs is significantly thicker than at the poles ($t_{(2),220} = 3.728$, $p<0.001$; $t_{(2),182} = 10.874$, $p<<0.001$). The equatorial shell from hatched eggs is significantly thinner than the same section of fresh, unhatched eggs ($t_{(2),201} = 5.278$, $p<<0.001$) but the poles are thinner in fresh eggs ($t_{(2),201} = 3.046$, $0.002<p<0.005$) (Table 3.8).

The shell of egg 4607 is thickest around the equator and thins rapidly towards the poles. Both poles are of similar thickness (Fig. 3.11). Although no other eggshell separated completely from the membrane, the variation in shell thickness shown in egg 4607 is probably typical because the equator of most eggs is thicker than the poles (Table 3.8). An occa-

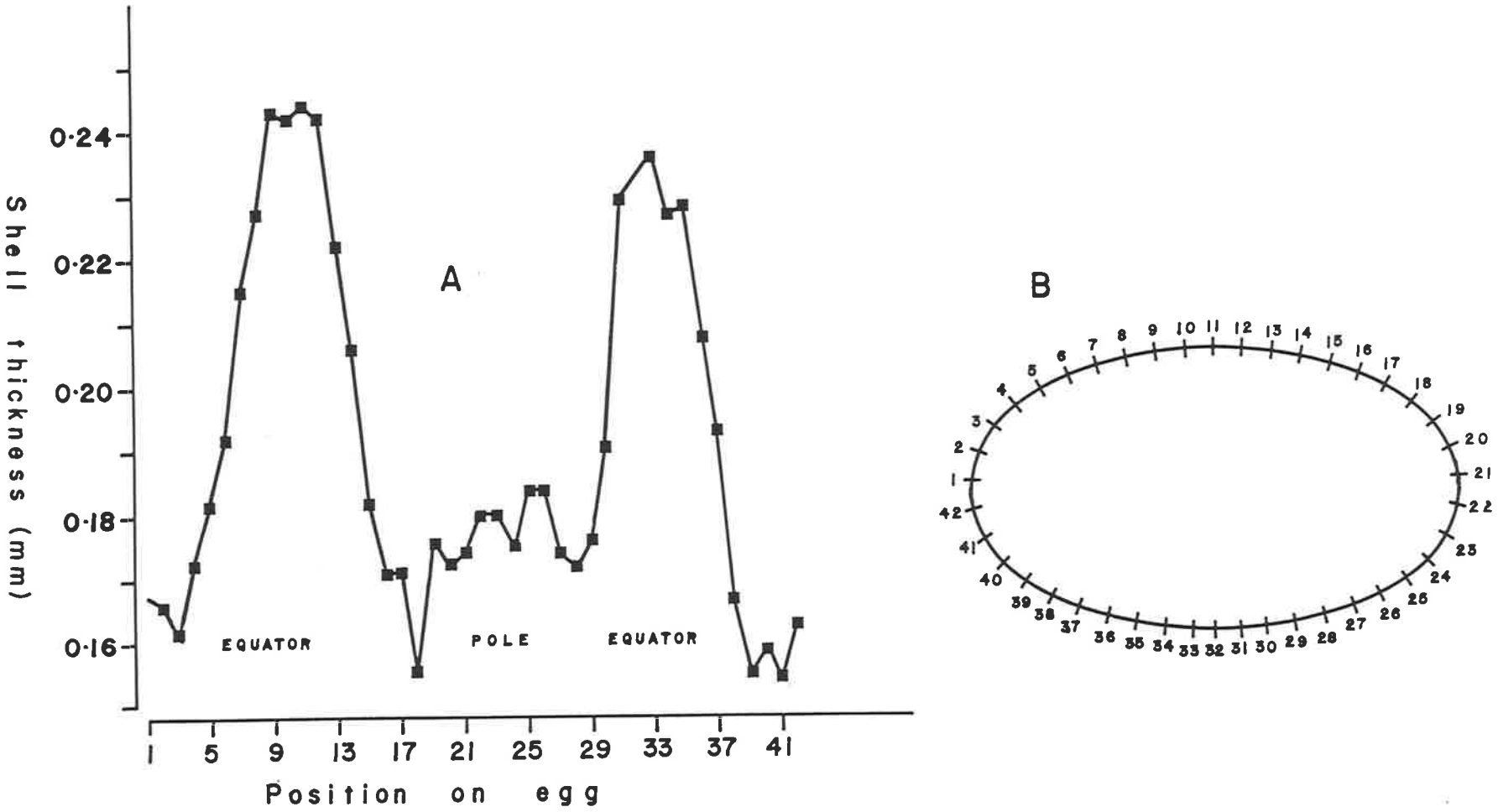


FIGURE 3.11 A. THICKNESS OF SHELL OF EGG NO.4607 PLOTTED AGAINST POSITION OF MEASUREMENTS.
B. SCHEMATIC DIAGRAM OF EGG NO.4607 INDICATING POSITION OF EACH MEASUREMENT PLOTTED IN FIGURE 3.7A.

TABLE 3.8 THICKNESS OF EGGSHELL FROM THE EQUATOR AND POLES OF FRESH AND HATCHED EGGS

Eggshell thickness (mm)	$\bar{x}$	S.D.	n	Range
Fresh				
Equator	0.191	0.024	92	0.128 - 0.262
Poles	0.155	0.022	92	0.101 - 0.202
Hatched				
Equator	0.177	0.014	111	0.153 - 0.208
Poles	0.166	0.028	111	0.119 - 0.257

sional egg is encountered with a shell that is thicker at the poles than the equator. This is due to calcareous ridges and bumps on the outer surface of the shell at the poles.

There is only minor variation in thickness (<2.5%) around the equatorial plane of the eggshell (Fig. 3.12).

The shell membranes are as thick at the equator as the poles ($t = 0.152$, $p>0.50$) (Table 3.9). Large standard deviations indicate a wide variation in the results.

TABLE 3.9 THICKNESS OF SHELL MEMBRANE (mm) AT THE EQUATOR AND POLES OF EIGHT EGGS

	Mean	S.D.
Poles	0.056	0.023
Equator	0.058	0.029

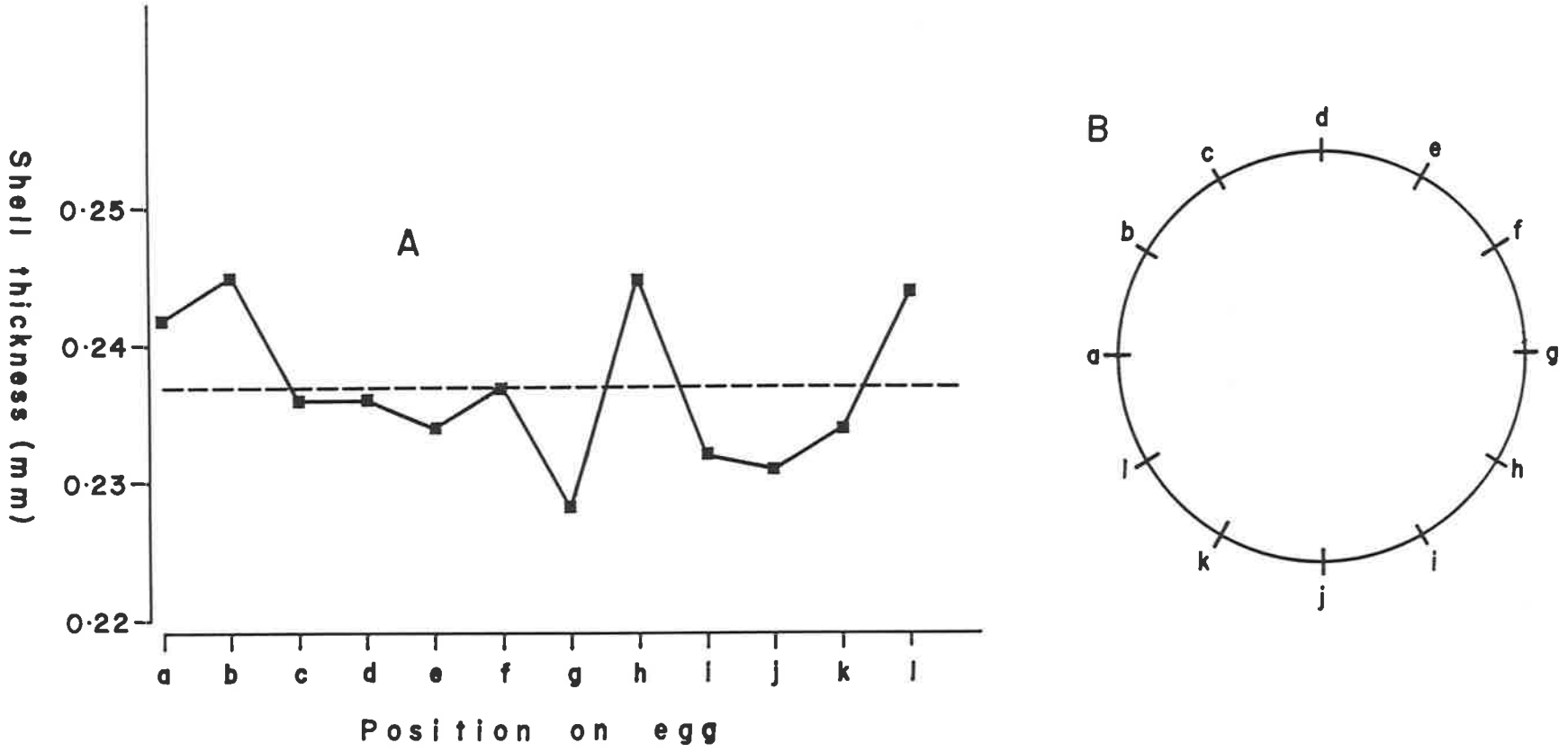


FIGURE 3.12 A. THICKNESS OF EGGSHELL OF EGG No.4607 MEASURED IN THE MIDDLE OF THE EGG PLOTTED AGAINST THE POSITION OF MEASUREMENTS. POSITION 1 IS THE SAME AS POSITION 11 IN FIGURE 3.11. BROKEN LINE REPRESENTS THE MEAN SHELL THICKNESS.

B. SCHEMATIC DIAGRAM OF EGG No.4607 INDICATING POSITION OF EACH MEASUREMENT PLOTTED IN FIGURE 3.12A.

3.3.7 Structure of Eggshell and Membranes

The eggshell is composed of a thick inorganic section with a complex of membranes consisting of layers of fibres on its inner surface (Figs. 3.14A,C,D, 3.17B). The inorganic section is of the typical form shown in other species with well organised shells (e.g. *Trionyx spiniferus* (M. Packard & Packard, 1979) and *Chrysemys picta* (M. Packard *et al.*, 1982a)) made up of crystalline units that arise from organic centres of crystallization (Fig. 3.14C), or cores, attached to the outer surface of the shell membrane (Figs. 3.16A,B). Crystal growth occurs in all directions from the cores. The inward growth of crystals is small (Fig. 3.14C) and terminates in conical tips (Fig. 3.15A,B) that are firmly attached to the shell membranes. Outward growth is more extensive (Figs. 3.14C,D, 3.17B), with individual shell units maintaining their integrity to the surface of the egg to form columns (Figs. 3.14C-F). There is no evidence of a surface of tangentially orientated crystals as described by Becking (1975) for avian eggs and Ferguson (1982) for eggs of the alligator.

The shell units are not regularly shaped in tangential view and they vary considerably in size (Figs. 3.14E,F). The maximum diameters at the equator exceed ($\bar{x}$ = 181 ± 63μm, n = 82, range = 45 - 357μm) those at the poles ($\bar{x}$ = 115 ± 33μm, n = 30, range = 51 - 169μm) as measured from the micrographs.

The pores, as revealed by casts (Fig. 3.17C) and radial sections (Fig. 3.17B) are of the Type 1a (outer orifice open, unbranched) of Board *et al.* (1977) at the equator and Type 3a(ii) (outer orifice capped with inorganic material, unbranched) at the poles. Measurements on 20 casts show that pores are hourglass-shaped with flat tops and bottoms (Fig. 3.13). This is a simplification because most of the pores are not round in section especially at the base where the adjacent shell units meet

(Fig. 3.17C). The position of the pores in relation to the shell units (Figs. 3.14E, 3.17A,B) are the same as in other species of animals with calcium-shelled eggs, i.e. usually between four shell units (Tullett, 1975; Becking, 1975; M. Packard & Packard, 1979; Figs. 3.14E,F, 3.17E). Shell units without pores generally meet in groups of three (Tullett, 1975; Fig. 3.14E). Counts under the binocular light microscope indicated that there are very few pores at the poles. Photomicrographs of the outer surface of the shell show a considerable growth of small inorganic knobs in the sulci between shell units on the poles of eggs (Fig. 3.14F) but not at the equator (Fig. 3.14E). These may have obscured pores from view under the light microscope, but may not have prevented them from functioning.

An examination of the conical tips of the shell units on the inner surface of the shell (Figs. 3.15A,B) shows that there is considerable growth of calcareous deposits at the poles of the egg but not at the equator. These deposits are similar to those on the outer surface at the poles (Fig. 3.14F). It is not known why or how they form or what their function is.

On hatching, the shell membranes become detached from much of the shell, taking with them the conical tips of the shell units together with many of the inorganic cores (Figs. 3.14D, 3.15C,E,F). The conical tips appear to be attached to the outer surface of the shell membrane of hatched eggs (Fig. 3.15F).

Measurements of eggshell thickness were made from 80 micrographs of casts of the eggshell from 9 eggs. These measurements (Table 3.10) are within the range measured using the screw micrometer (Table 3.8). There is no difference in mean shell thickness at the equator of hatched and

fresh eggs. The range of eggshell thickness is large. Although the shell of hatched eggs was expected to be thinner because of the removal of the conical tips with the outer shell membrane, presumably influenced by the embryo (M. Packard & Packard, 1979), this is not indicated by the measurements from the micrographs (Table 3.10). However the large number of micrometer measurements (Table 3.8) indicate this to be an artifact of the measuring technique. The conical tips are clearly visible on the inner surface of fresh eggshell (Figs. 3.14C, 3.15A,B) but are absent from hatched eggs (Figs. 3.14D, 3.15C,D,E). The organic cores are absent from much of the shell of hatched eggs (Figs. 3.14D, 3.15D),

TABLE 3.10 THICKNESS OF EGGSHELL AT THE EQUATOR OF HATCHED AND FRESH EGGS AND THE POLES OF HATCHED EGGS MEASURED FROM MICROGRAPHS

Eggshell thickness (mm)	$\bar{x}$	S.D.	n (measurements)	Range	Eggs examined
Fresh					
Equator	0.167	0.037	116	0.112 - 0.258	3
Hatched					
Equator	0.170	0.026	137	0.124 - 0.236	4
Poles	0.157	0.018	29	0.116 - 0.180	2

Careful dissolution of the inorganic shell with various concentrations of HCl and acidified alcohol failed to reveal a cuticle on the outside of the eggshell.

Light microscopy showed 3.456 ± 4.256 pores per mm^2 at the equator and 0.032 ± 0.112 per mm^2 at the poles of the shell (n = 13). Figure 3.11A shows that the shell at the poles of the egg is much thinner than

at the equator. It is assumed that the pore density from the equator (i.e. 3.456 mm^{-2}) is uniform over the middle part of the egg, as delimited by Figure 3.11A, and similarly the pore density (i.e. 0.032mm^{-2}) is uniform over the polar regions. On the assumption, based on Figure 3.11A,B that the poles together cover 50% of the shell surface and the equator the other 50%, pore number is calculated to be 3,673 ± 4,599 over the surface of the egg, but the range is great (103 - 17,720).

The diameter of 20 pores was measured from micrographs of the casts at 10 evenly spaced points across the eggshell (Fig. 3.13). The minimum diameter is 18.5 ± 12.7μm. Assuming this to be the effective pore diameter, the effective pore area of an egg with 3,673 pores is 0.987 mm^2, or 0.043% of the mean shell area.

Although the conical tips of the shell units are absent from the whole of the inner surface of the shell of hatched eggs, the organic cores remain in some areas but not others (Figs. 3.15D,E). The cores are removed in lines running across the inner surfaces of the shell, and shell units appear to be differentially "corroded" along these same lines (Figs. 3.15D,E). These lines of corrosion range from 65 - 103μm wide (from five micrographs).

Casts made from eggshell with membranes attached (Fig. 3.14B) indicate that the calcareous shell is about three times as thick as the shell membrane, which confirms the micrometer measurements (Tables 3.8, 3.9). In Figure 3.14A the calcareous shell appears to be equal in thickness to the membrane but this is not really the case because the membranes, but not the shell, were cut obliquely.

Electron micrographs of torn edges of the shell membranes indicate

that they are composed of five discrete layers of fibres (Fig. 3.16C). It is not possible to tell how the layers of fibres adhere to each other but no fibres are observed crossing from one layer to the next. Each of the five fibre layers consist of less distinct but apparently discrete sublayers (Fig. 3.16D). The fibres are not cylindrical (Fig. 3.16D) and vary considerably in their maximum diameter (Fig. 3.16C). Layers are numbered 1 - 5 with 1 being the outermost layer adjacent to the shell and 5 being the inside layer. The mean diameters of fibres taken from micrographs clearly show that layers 1 and 4 contain much larger fibres than the other layers and layer 5 contains the smallest fibres (Table 3.11). However layers 2, 3 and 5 contain fibres of similar sizes. When cut with an edge-cutting disc the five layers divide into two discrete units (Fig. 3.14A) which presumably correspond to the two shell membranes in avian eggs. It could not be determined between which layers of fibres the junction between these membranes occurs.

TABLE 3.11

Layer	1	2	3	4	5
$\bar{x}$ (µm)	2.6	1.3	1.0	4.5	0.9
S.D.	1.5	0.5	0.5	1.9	0.3
n	30	20	20	10	15
Range (µm)	1.1 - 8.9	0.9 - 3.0	0.4 - 1.9	2.6 - 8.6	0.4 - 1.2

Diameter of fibres (mean maximum (µm), S.D. and range) measured in the 5 discrete layers of the shell membrane. Layer 1 is adjacent to the shell and 5 is the innermost layer.

The casts show many projections pointing towards the surface of the eggshell from the valleys between the shell units, and presumably passing between them (Fig. 3.17D). They appear to be incipient pores that become

closed as the shell was calcified rather than pores into which the Spurr's resin did not fully penetrate. Similar incipient pores occur in avian eggs (Fujii, 1974).

The decalcified membrane of an egg that died early in development shows an irregular scattering of organic knobs which presumably represent the organic cores of the shell units (Figs. 3.16A,B). These knobs are firmly attached to the outer surface of the shell membrane and presumably have fibrous connections to it, as in other species (Fujii & Tamura, 1970; M. Packard & Packard, 1979).

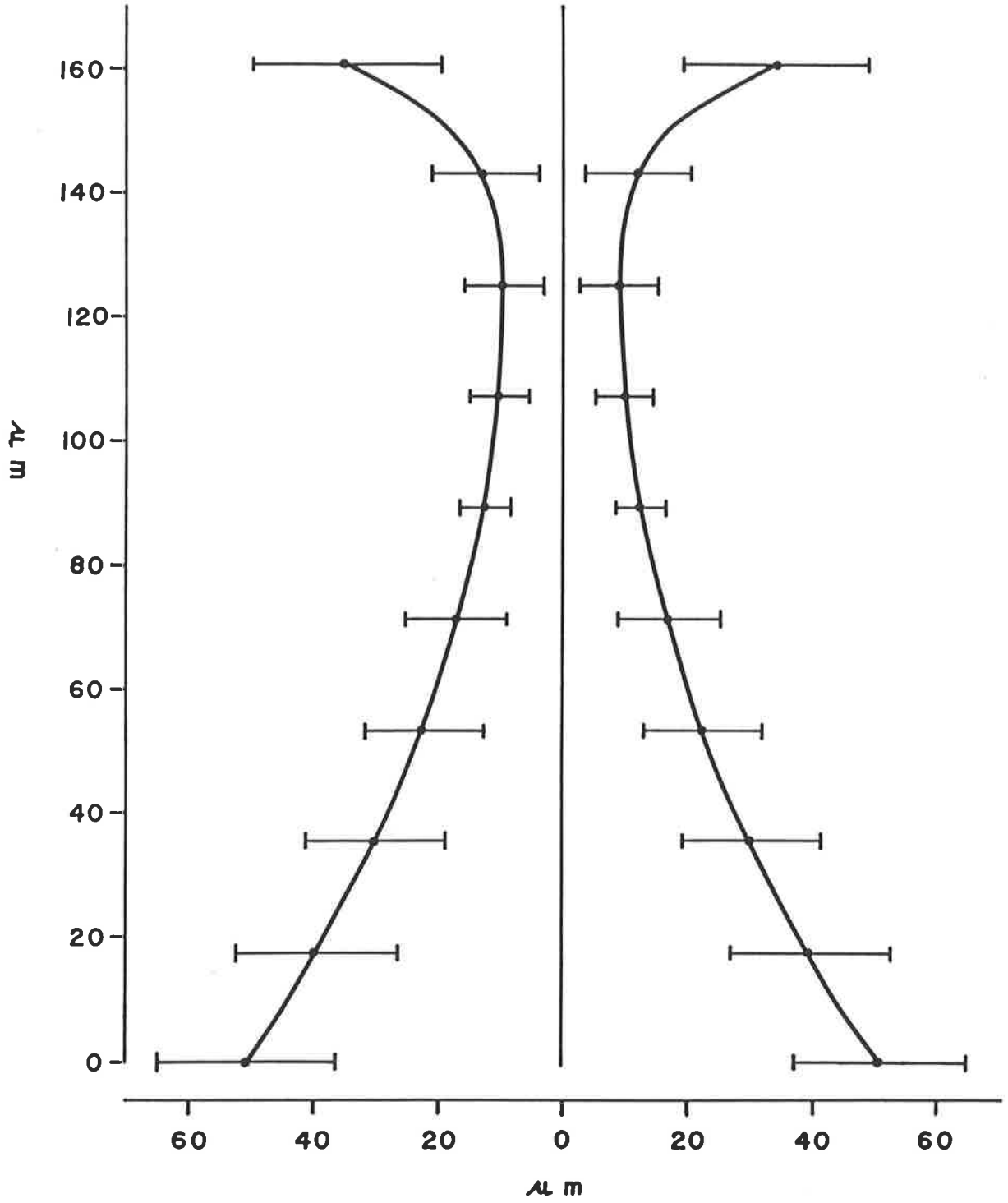


FIGURE 3.13

MEAN DIAMETER OF 20 PORES TAKEN FROM CASTS AT TEN POINTS FROM THE OUTSIDE OF THE SHELL (top) TO THE INSIDE (bottom).

FIGURE 3.14

A. Radial section of eggshell and membranes illustrating the fracture (m) of the shell membrane into two units.

B. Cast of shell with membranes attached indicates the relative thickness of each.

C. Radial section of calcareous shell of an undeveloped egg with organic matter dissolved away. Conical tips (t) of shell units with centres of crystalization (c) are clearly visible.

D. As in C but from a hatched egg. Conical tips and cores have been separated with the shell membranes during development.

E. Tangential view of the outside of shell from the equator of the egg. Individual shell units (u) are clearly visible as are many pores (p).

F. Similar view as E but from the pole. The surface of the shell and pore openings appear to be covered with secondary calcareous deposits (d).

Scale bars on all plates represent 100 μm.

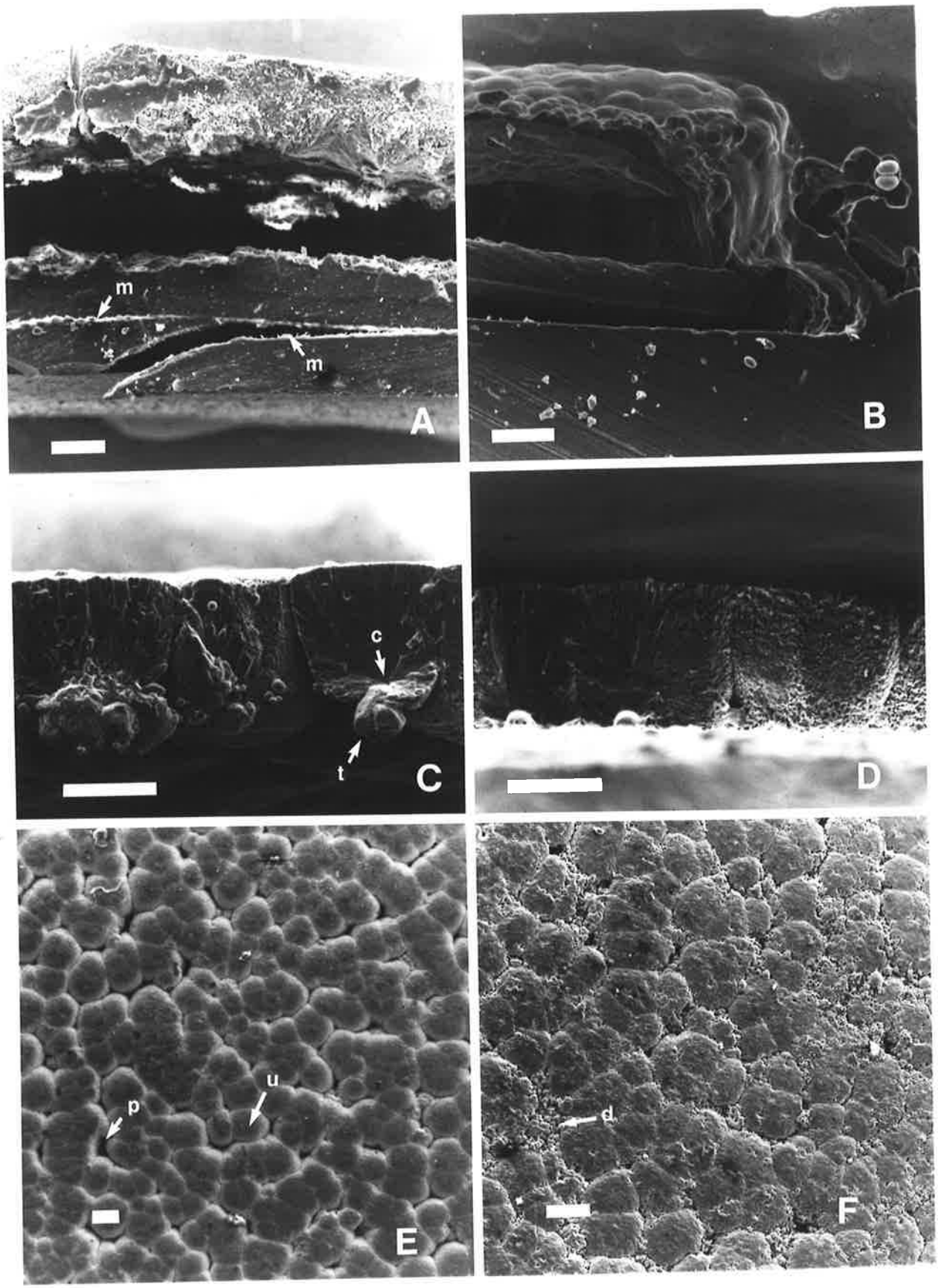

m
m
A
B
c
t
C
D
p
u
E
d
F

FIGURE 3.15

A. View of inner surface of shell from the equator of an undeveloped egg showing conical tips (t) of shell units.

B. Same view as A but from a pole of the egg showing secondary calcareous deposits (d).

C. Same view as A but from an egg that has hatched showing that the conical tips and organic cores have pulled away from the shell with the shell membrane during development.

D. Inner view of hatched eggshell showing complete removal of the conical tips of shell units, the lines of intense "corrosion" (l) of the calcium, and removal of the organic cores.

E. Closer view of line of corrosion from top right of D.

F. Outer surface of shell membrane of an egg that hatched. The membrane came away from the shell naturally and the centres of calcification of the shell are clearly visible on the surface. The fibres (f) of the outershell membrane are visible in places.

Scale bars A, D and E represent 100 μm and on B, C and F represent 10 μm.

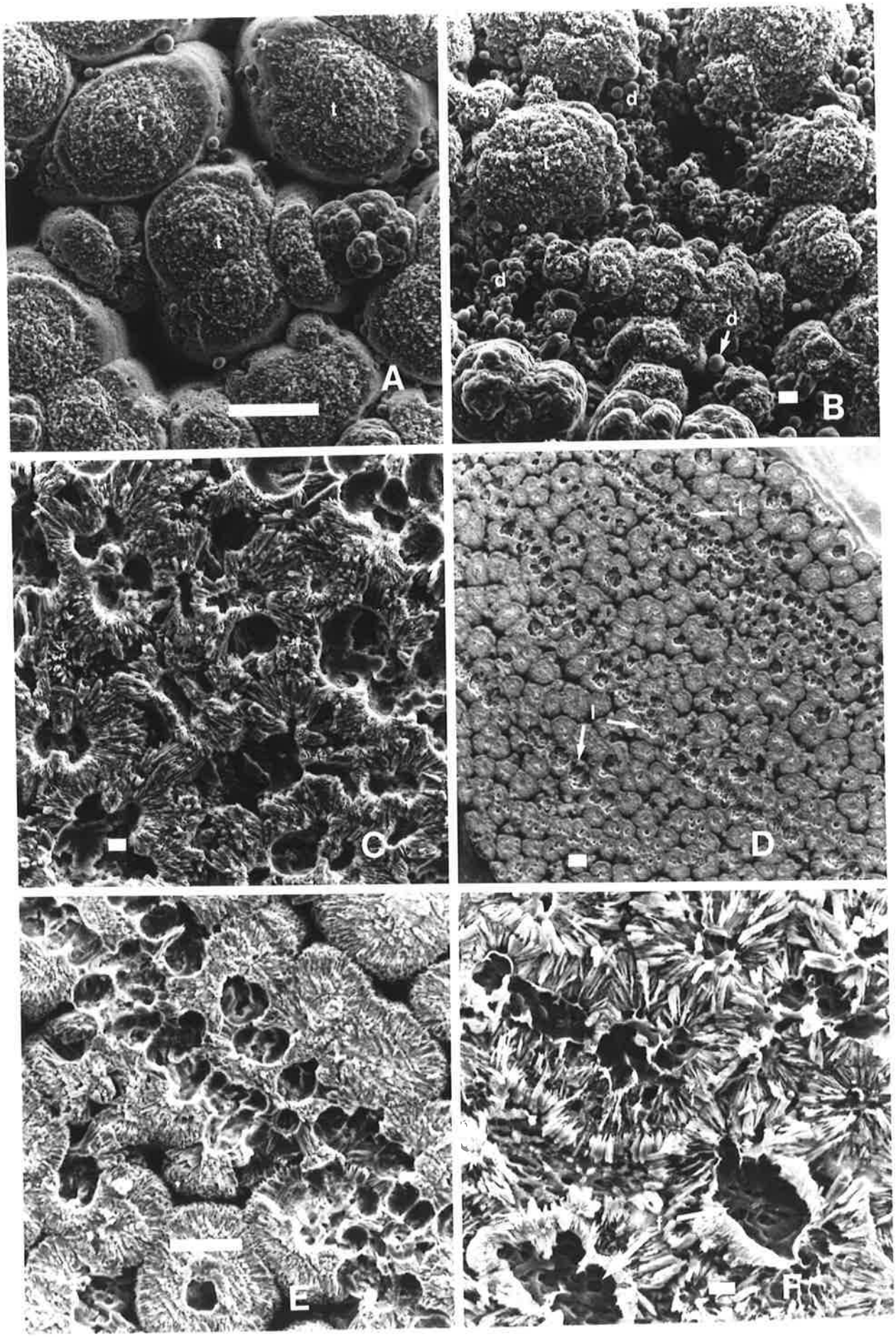

t
t
t
A
d
d
d
B
C
t
D
E
F

FIGURE 3.16

A. Possible organic cores (o) attached to the outer surface of a decalcified shell membrane from an egg that died early in its development.

B. Closer view of one of the structures shown in A.

C. Torn edge of shell membrane showing five discrete layers of fibres. Top layer is adjacent to the shell and bottom layer is adjacent to the egg contents. Each layer appears to consist of fibres of different diameter.

D. Edge of layer 3 showing that it appears to be made up of separate sublayers (s). Note fibres are not cylindrical.

E. Closer view of fibres in layer 1 showing variation in fibre diameter and the orientation of the fibres in one plane only.

Scale bars A and C represent 100 μm and B, D and E represent 10 μm.

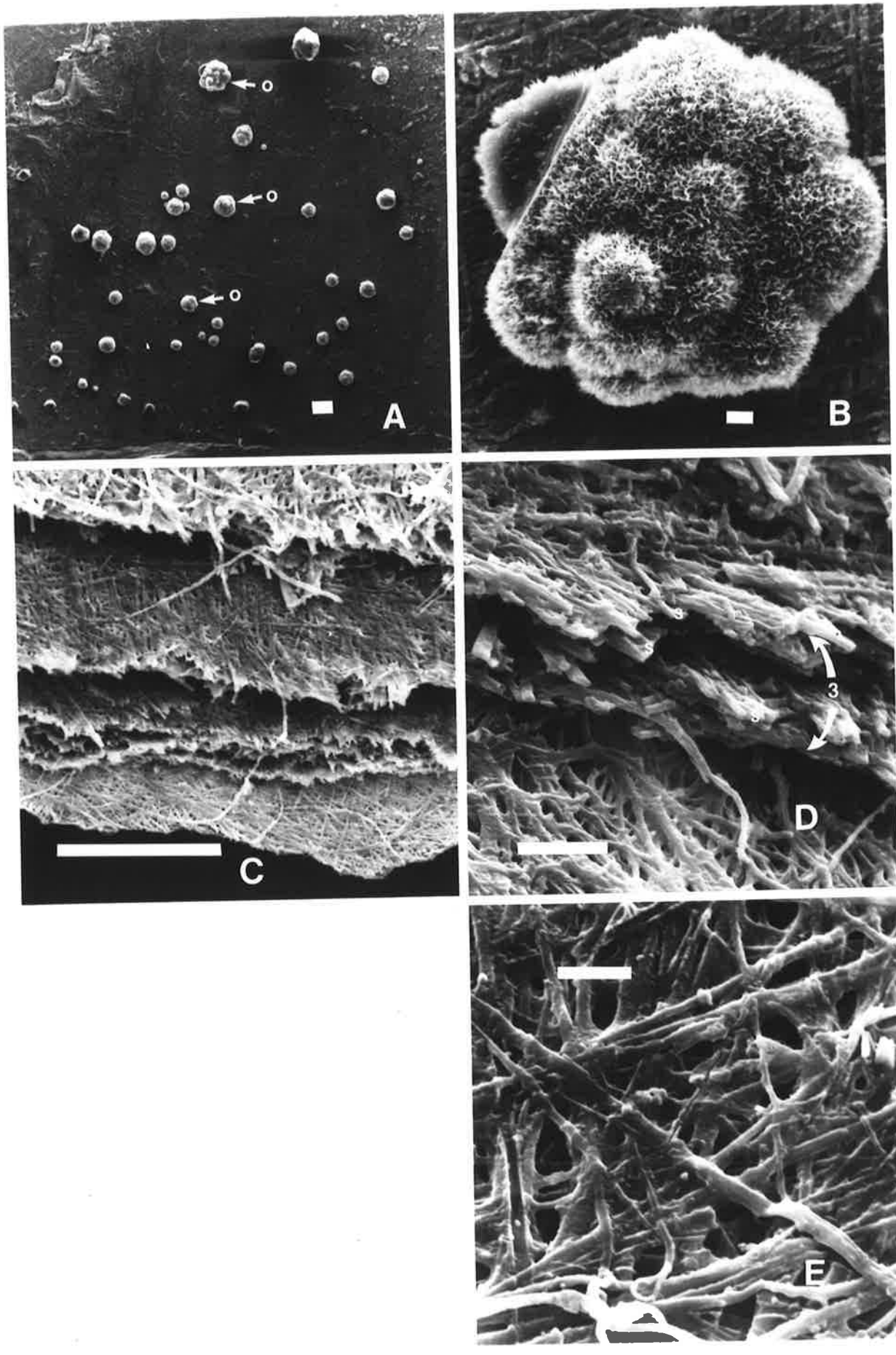

o
o
o
A
B
C
3
D
E

FIGURE 3.17

A. Opening of a pore on the surface of an eggshell. It is bounded by four shell units (u).

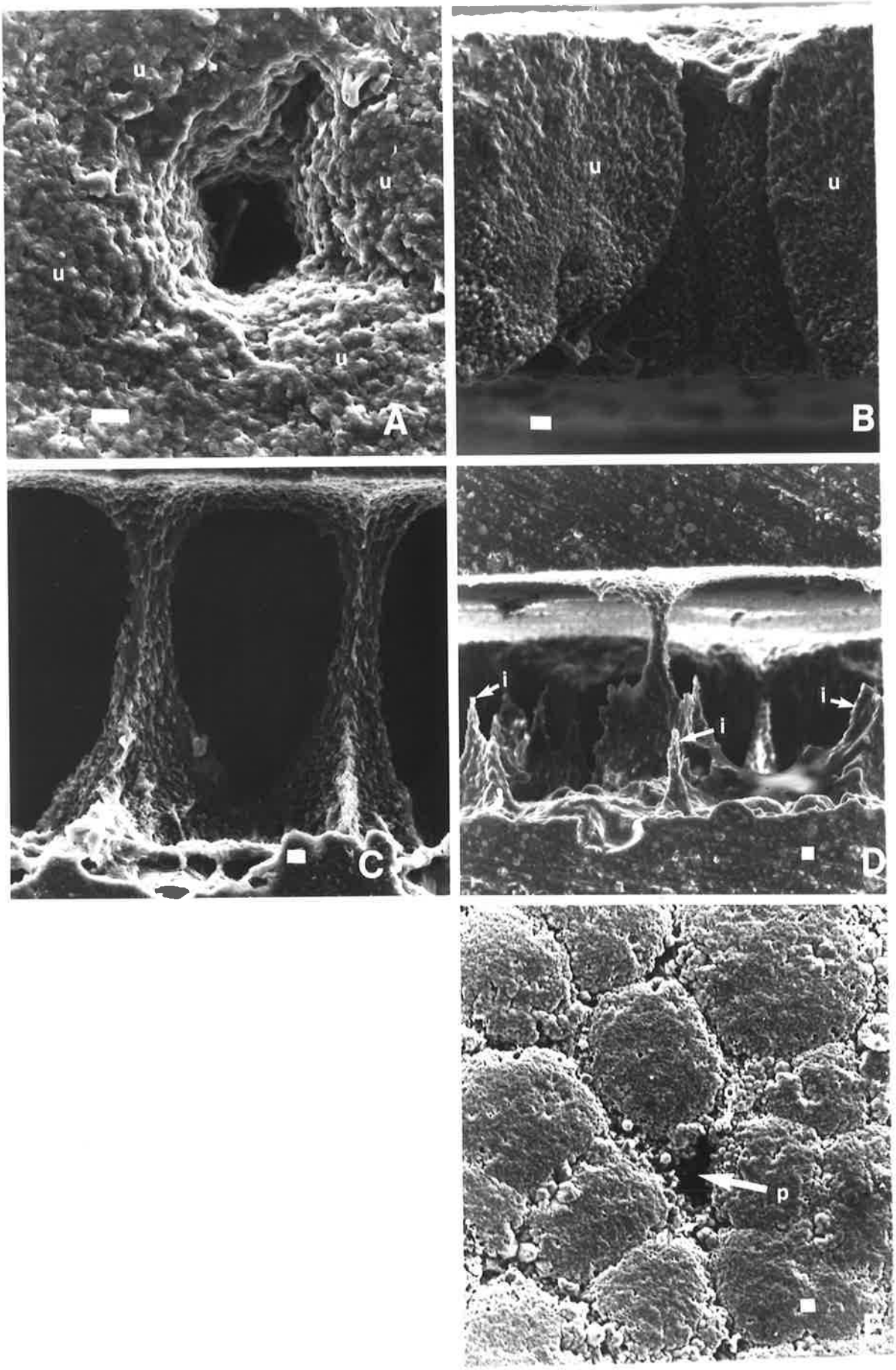
B. Radial section of pore showing its relationship to the shell units (u). Note also the texture of the shell unit in the pore.

C. Cast of two pores. Outside of shell to top of plate. Note that the texture of the surface of the cast reflects that in B. Pores typically have a "buttressed" appearance on the inner surface of the shell where the shell units meet.

D. Cast of shell showing many "incipient" pores (i) appearing as projections from the inner surface of the shell.

E. Pore opening (p) on surface of a pole of an egg showing it to be severely occluded by secondary calcareous deposits (d).

All scale bars represent 10 µm.


u
u
u
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u
A
u
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C
i
i
i
D
p

3.4 DISCUSSION

3.4.1 Linear Dimensions and Mass of Eggs

Mass and breadth, and mass and length, are exponentially related in the eggs of *E. macquarii* (equations 3.2, 3.3). Egg mass is linearly related to egg breadth and length in *Terrapene carolina* (Tucker *et al.*, 1978). These results are not surprising since egg mass is a function of size. Egg length and egg breadth (Fig. 3.1) are positively correlated in *E. macquarii* which indicates that, as egg size increases, the shape of the egg, and not the volume, is conserved (Preston, 1969). Because the regression of elongation against egg breadth (Fig. 3.4) has a slope of zero, the conservation of shape as egg size increases is confirmed.

There is more variation in egg length than egg breadth within clutches in *E. macquarii*. In *Chrysemys picta*, variation in egg length is significantly greater than in egg breadth in 70% of clutches (Tucker *et al.*, 1978) and egg diameter is very similar within a clutch of the spherical eggs of *Chelydra serpentina* but clutches vary greatly from each other (Yntema, 1968).

There is more variation in the dimensions of eggs from different clutches than those from the same clutch in *E. macquarii*. In *Malaclemys terrapin* variation between clutches accounts for 74% of the variation in egg length and 82% in egg breadth (Montevecchi & Burger, 1975). Less variation in egg breadth than length and elongation in *M. terrapin*, between and within clutches, is probably a result of the physical constraints imposed by the oviducts of the female (Montevecchi & Burger, 1975).

3.4.2 Female Tortoise : Egg Relationships

When comparing the size of female tortoises to the size of their eggs, most authors use linear dimensions and not weights (e.g. Cagle, 1950; Ernst, 1970). In this study exponential equations best fitted the points relating egg weight to tortoise weight ($r^2 = 0.434$, n = 32, equation 3.5) and egg weight to the curved plastron length of the tortoise ($r^2 = 0.413$, n = 32, equation 3.6).

Comparison of plastron length of female tortoises with breadth and length of their eggs (Figs. 3.6, 3.7) shows a wider scatter of points in the length than the breadth comparison. There is a correlation between egg breadth and plastron length (equation 3.7) but no significant correlation between egg weight and plastron length (equation 3.8). Plastron length is positively correlated with egg breadth in both *T. carolina* and *Chrysemys picta* but there is no significant correlation between plastron length and egg length in either species (Tucker *et al.*, 1978).

Egg breadth may be less variable than egg length because the maximum breadth of the egg is constrained by the dimensions of the pelvic canal, reducing the likelihood of producing an egg too large to lay (Tucker *et al.*, 1978). This may be universally true because even double-yolked chicken eggs are about as wide as normal eggs, but they are much longer (Preston, 1969). However, this merely explains the lack of variation in egg breadth and not the large amount of variation in egg length. Such comparisons assume that carapace length and body weight are related to the dimensions of the pelvic canal. It also assumes that egg breadth is continually increased to match pelvic canal dimensions as the animal grows. This is supported by the fact that egg breadth and plastron length are significantly correlated in *E. macquarii* (equation 3.6) and in *C. picta* and *T. carolina* (Tucker *et al.*, 1978).

The larger eggs of *E. macquarii*, like many other species (Yntema, 1968, 1970; Tucker *et al.*, 1978; Cox & Marion, 1978; Swingland & Coe, 1979), give rise to larger hatchlings, i.e. the ratio of hatchling weight to fresh egg weight (48.06%) is constant over a wide range of egg sizes and is similar to two other Australian short-necked chelids reported by Ewert (1979). However, the ratio in *E. macquarii* is amongst the lowest of 55 taxa from nine families on which he reported (four are lower). Larger hatchlings are thought to have a selective advantage because they are better able to exploit resources and evade predation (Froese & Burghardt, 1974; Tucker *et al.*, 1978; Swingland & Coe, 1979). If large egg size is important and egg breadth is limited by the pelvic canal of the female one would expect elongation to be maximized. The positive correlation between egg length and breadth confirms that egg shape is conserved as the eggs become longer as the mother grows, which suggests that elongation is maximised. Although mean elongation is only 1.64, one clutch had eggs with an elongation of 1.94, which indicates that *E. macquarii* has the capacity to produce longer eggs. The elongation of 53% of the chelonian taxa listed by Preston (1969) and Ewert (1979) was greater than or equal to 1.64.

All the characteristics being considered (egg length, pelvic canal size and therefore egg breadth, elongation, egg size to hatchling size ratio and clutch size) are variable and therefore potentially subjected to selection. Different selective pressures may operate in opposite directions on one characteristic. Chelonians are generally long-lived and have a long reproductive life (Auffenberg & Iverson, 1979; Graham, 1979). Consider, for example, a particular female tortoise. Early in her reproductive life her eggs are small and the resulting small hatchlings have a reduced rate of survival. As egg shape (elongation) is maintained, the female gives rise to larger, fitter hatchlings as she

grows. Therefore the hatchlings she produces later in her life are selected in the population even though their smaller sibs, produced earlier in her life, are less fit. The larger hatchlings in turn produce small, unfit hatchlings early in their lives and larger, fitter ones later. In this case there must be some advantage to maintaining elongation through a range of egg sizes to the detriment of the hatchlings arising from small eggs or else eggsize would be maximised and elongation would fall throughout the life of the female. This situation would also select for greater longevity and size.

I suspect that for the Lake Bonney population of *E. macquarii*, the number of eggs per clutch and body mass is positively correlated because egg mass is positively correlated with female size and there is no evidence to suggest that larger females lay fewer eggs in a clutch. There is no significant relationship between mean egg mass and the number of eggs in a clutch of wild collected eggs ($r^2 = 0.084$, $n = 16$). Considering the statement of Gibbons and Tinkle (1969) that body mass and clutch size within a population are independently influenced, I would expect a wide degree of variation in the Lake Bonney data. Only 9% of the variation in the clutch size to plastron length relationship and 13% of the clutch size to carapace length relationship are attributed to the relationship in *Chrysemys picta*, the rest of the variance is due to other factors (Tinkle *et al.*, 1981). Other influences that may be significant are climate, population density and genetic factors (Cagle, 1954; Gibbons & Tinkle, 1969; Tucker *et al.*, 1978; Swingland & Coe, 1979). As there is no information available on any of these parameters in *E. macquarii* these ideas cannot yet be tested.

3.4.3 Water Content of Eggs and Hatchlings

The percentage of wet mass of various parts of the eggs (shell, albumen, yolk) of *E. macquarii* (Table 3.4) falls within the range of values reported by Ewert (1979) for 15 species of the Chelonia. The water content of whole eggs and hatchlings is similar in *E. macquarii* (Table 3.6).

In the eggs of *Chelydra serpentina* 70.9% of the fresh egg and 79.0% of the hatchling is water (Lynn & von Brand, 1945; von Brand & Lynn, 1947). Although the water content of the hatchlings are similar more water is contained in the hard-shelled (or hard expansible-shelled) eggs of *E. macquarii* than the soft-shelled eggs of *C. serpentina*, which absorb water during incubation (G. Packard *et al.*, 1980, 1981b, d). Thus, eggs that readily absorb water during incubation may begin with relatively less water than hard-shelled eggs that do not absorb water.

3.4.4 Thickness of Eggshell and Membranes

The eggshell of *E. macquarii* is thicker in the middle of the egg than at the poles (Fig. 3.10) but is of relatively uniform thickness in a latitudinal plane through the egg (Fig. 3.11). The membrane thickness is uniform over the whole egg. Variations in the shell thickness of avian eggs may help provide maximum strength while maintaining minimum thickness (Tyler, 1969). However, eggs of *E. macquarii*, like other chelonians, are incubated in subterranean nests in which there is little requirement for strength. During oviposition the shells of some eggs are cracked when they hit one another, but the membranes remain intact and the hatching success is not reduced. Many chelonian species have less calcium in the shell, resulting in soft-shelled eggs, so the reason for *E. macquarii*, and other species, having such thick shells remains to be investigated.

It is likely to be related to the hydric conditions experienced during incubation. The eggs of *E. macquarii* hatch successfully in incubation environments far drier than those that result in successful hatching of the soft-shelled eggs of *Chrysemys picta* (G. Packard *et al.*, 1981a, Chapter 6), for example. In some parts of the world, species that lay soft-shelled eggs occur sympatrically with others that lay hard-shelled eggs, e.g. South Carolina (Gibbons *et al.*, 1982). The shell type often follows taxonomic lines (Ewert, 1979), so areas in which both hard- and soft-shelled species occur probably represent radiations of taxa with different eggshells into mutually favourable environments.

3.4.5 Structure of Eggshell and Membranes

The eggs of *E. macquarii* are hard-shelled and, like other chelonians, composed of crystals of calcium carbonate assumed to be mainly in the form of aragonite (Young, 1950; Erben, 1970; Erben & Newesely, 1972; Solomon & Baird, 1976; M. Packard *et al.*, 1982a) formed around an organic matrix (Tyler & Geake, 1953a; Tyler, 1969; Becking, 1975). The shells are penetrated by pores which allow gaseous diffusion and the flow of liquid water (see Chapters 5 & 6). The eggs of *E. macquarii* crack towards the end of incubation (Fig. 6.9) (as in some other chelonians: Ewert, 1979) if incubated on moist substrates, but this was not observed in the field. When incubated in substrates of low water potential a net loss of water occurs (Chapter 6) and an airspace forms in the albumen. An airspace forms between the shell membranes in most avian eggs but only occasionally in infertile or dead eggs of *E. macquarii*.

The structure of the eggshell and membranes of *E. macquarii* supports the suggestion that they are intermediate between soft- and hard-shelled eggs. The structure of the shell surface and individual shell units

(Fig. 3.13D,E) is very similar to that of *Chrysemys picta*, which lays soft-shelled eggs (M. Packard *et al.*, 1982a). The calcareous shell of soft-shelled chelonian eggs is about as thick as the underlying membranes whereas that of hard-shelled eggs is much thicker (M. Packard *et al.*, 1982a). In *E. macquarii* the shell is about three times as thick as the membrane (Tables 3.8, 3.9). Like hard-expansible shelled eggs, which are intermediate between hard- and soft-shelled eggs (Ewert, 1979), the eggs of *E. macquarii* will expand and crack if incubated in moist conditions (Fig. 6.9) but, like hard-shelled eggs, the hatching success and size of hatchlings are uninfluenced by the hydric environment during incubation in *E. macquarii* (Chapter 6).

The structure of the shell and membranes indicates that the process of shell formation in the eggs of *E. macquarii* is similar to other species of chelonians (M. Packard & Packard, 1979) and therefore of birds (Fujii & Tamura, 1970; Simkiss & Taylor, 1971; Fujii, 1974).

Embryonic chelonians absorb calcium from the eggshell (Bustard *et al.*, 1969; G. Packard *et al.*, 1977a) for ossification of bones. This presumably weakens the attachment of the conical tips to the rest of the shell, allowing them to come away with the shell membranes during incubation. Micrographs of the inside of the shell of hatched eggs (Figs. 3.13D, 3.14C,D.E.) clearly show that the conical tips are no longer attached to the shell units but are attached to the shell membrane (Fig. 3.14F). Lines of corrosion on the inner surface of the shell of hatched eggs (Fig. 3.14D,E) have not been reported previously. Close examination of these lines (Fig. 3.14E) indicate that the calcium carbonate has been corroded at many places on the inside of the shell units and not just around the organic cores. As the maximum diameter of a chelonian erythrocyte is about 20μm (Saint Girons, 1970) the diameter of a blood

capillary can be assumed to be slightly greater than this. The lines of corrosion were three to five times this diameter wide so it is assumed that they represent lines of intense absorption of calcium into the embryonic blood adjacent to capillaries in the chorioallantoic membrane. This close agreement strongly suggests that the corrosion of the shell is influenced by the embryonic blood which lies very close to the shell membrane in the capillaries of the chorioallantoic membrane. Unfortunately the eggs of *E. macquarii* do not form an airspace like that in *Trionyx spiniferus* (M. Packard & Packard, 1979) so it is not known whether these lines are absent from places where the chorioallantoic membrane is not in close contact with the inner shell membrane. It is assumed that the mechanism of calcium absorption is the same as that in avian and *Trionyx* eggs, where water and CO_2 form carbonic acid, liberating calcium from the shell which is actively transported across the chorioallantoic membrane (M. Packard & Packard, 1979). There is some evidence that some other acid may also be involved in calcium resorption in avian eggs (Crooks & Simkiss, 1974).

The shell membranes in *E. macquarii* and the eggshell itself in the soft-shelled eggs of *Anolis* lizards consist of 5 main layers of fibres differing from each other in fibre diameter (Sexton *et al.*, 1979; Andrews & Sexton, 1981) which suggests that soft-shelled eggs may have developed from hard-shelled eggs by a reduction in the calcium carbonate component of the shell and an increasing importance of the shell membranes. Only 4 layers of fibres were noted in the soft-shelled eggs of the lizard *Callisaurus draconoides* (M. Packard *et al.*, 1982b) indicating further reduction. The fibre diameters in the shell and membranes of *E. macquarii* are similar to those in the shell membranes of birds (Becking, 1975) and alligators (Ferguson, 1982).

4

EGG METABOLISM

4.1 INTRODUCTION

The two basic patterns of O_2 consumption ($\dot{V}_{O_2}$) in incubating avian eggs (C. Vleck *et al.*, 1980) are also shown by different species of the Chelonia (Fig. 4.1). The $\dot{V}_{O_2}$ of altricial birds (Kendeigh, 1940; C. Vleck *et al.*, 1979) and the marine turtles, *Caretta caretta* and *Chelonia mydas*, (Ackerman, 1981b), increases exponentially throughout the incubation, and will be referred to as "exponential". The $\dot{V}_{O_2}$ of most precocial birds (C. Vleck *et al.*, 1980) and most chelonians (Hoyt & Albers, in press) increases exponentially for the first 80% of the incubation and then approaches an asymptote, and will be referred to as "sigmoidal". The sigmoidal pattern is modified in some birds with precocial hatchings, for example ratites, in which $\dot{V}_{O_2}$ declines prior to hatching (Hoyt *et al.*, 1978; D. Vleck *et al.*, 1980). The eggs of the freshwater turtle, *Trionyx spiniferus*, have a similar pattern (Hoyt & Albers, in press), here referred to as "peaked".

These patterns are correlated with developmental modes in birds (i.e. altricial and precocial) (C. Vleck *et al.*, 1980), but not reptiles. Reptilian embryos show all the patterns of $\dot{V}_{O_2}$ but they are all precocial, most species being independent of the parents from the time they hatch. Oxygen consumption reflects embryonic growth in both altricial and precocial birds (C. Vleck *et al.*, 1979), in turtles (Lynn & von Brand, 1945; Ackerman, 1981a,b) and in snakes (Dmi'el, 1970). Unlike precocial birds, altricial species hatch naked, with closed eyes and incapable of locomotion and thermoregulation (Kendeigh, 1939). Once tissue growth is essentially complete in precocial birds, the sensory, neuromuscular and thermoregulatory systems mature; this occurs during the plateau period and requires relatively little energy (C. Vleck *et al.*, 1979). Precocial hatchlings stimulated to emerge early show slight but significant deficiencies in motor behaviour because the time required to develop their

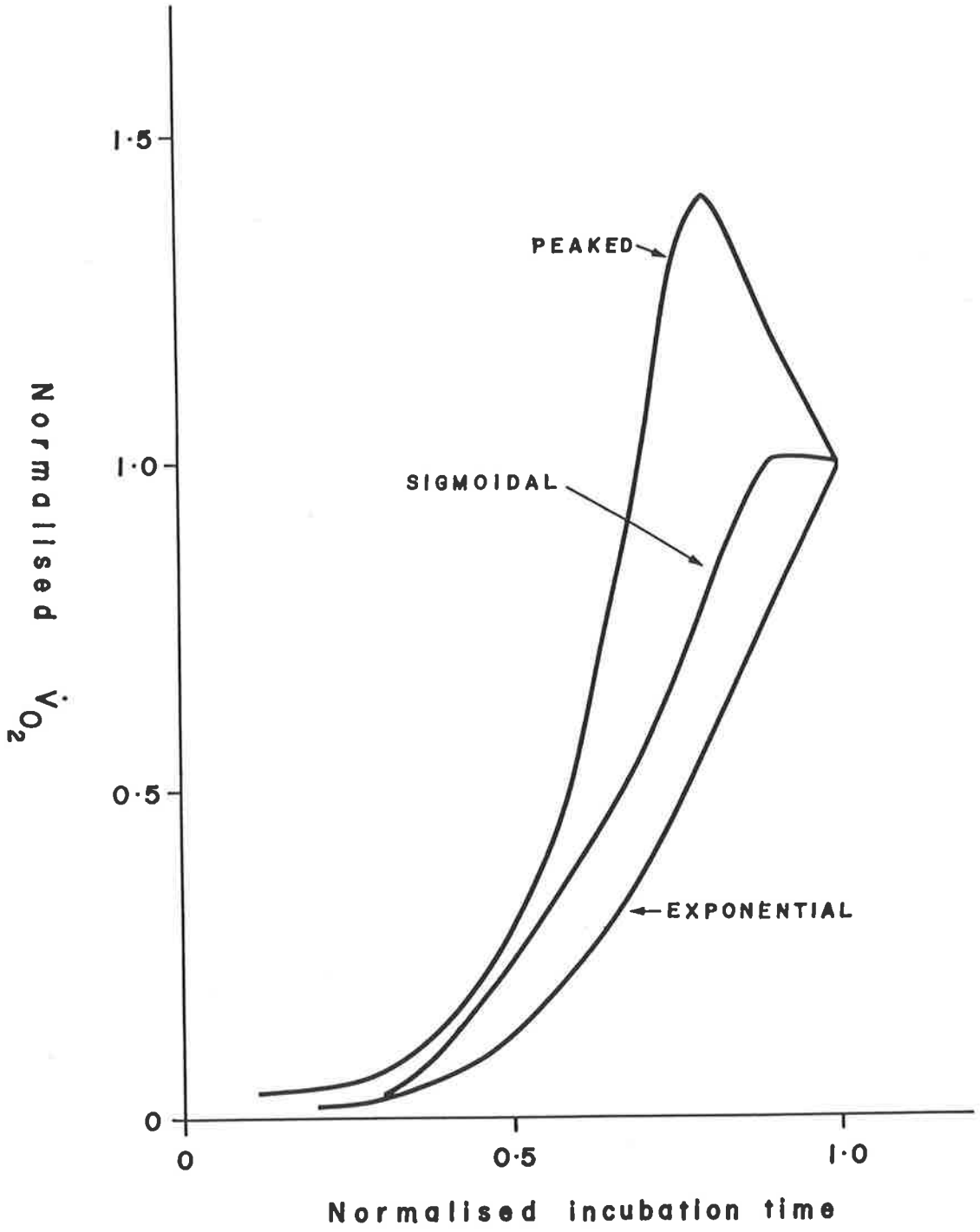


FIGURE 4.1

PATTERNS OF OXYGEN CONSUMPTION DURING INCUBATION FROM NORMALISED DATA.
Peaked - *Trionyx spiniferus*[1], ratites[2]; Sigmoidal - *Chrysemys concinna*[1], most precocial birds[2]; Exponential - *Chelonia mydas*[3], altricial birds[2].
1. Hoyt & Albers (in press), 2. C. Vleck *et al.*, 1980,
3. Ackerman, 1981b.

sensory and neuromuscular control is shortened (Vince & Chinn, 1971).

The pattern of O_2 consumption during incubation was investigated in the eggs of *E. macquarii* and compared to patterns found in other species of reptiles and birds. The rate of development of embryos was investigated to discover any correlations with the pattern of respiration (as in birds: C. Vleck *et al.*, 1980).

The respiratory quotient (RQ) is the ratio of O_2 consumed to CO_2 released during the metabolism of a given substrate, and the respiratory exchange ratio (RE) is the ratio of O_2 consumed to CO_2 expired during the respiration of an organism. If all the CO_2 released during metabolism is expired, then RQ equals RE. The respiratory substrate of avian and reptilian eggs has been assumed to be lipid (Ackerman, 1977), which has an RQ of 0.7 (Ricklefs, 1974). A mean RE of 0.7 has been determined for the eggs of birds (Kendeigh, 1940; Khaskin, 1961; Rahn *et al.*, 1974) and marine turtles (Ackerman, 1977) indicating that lipid is the respiratory substrate in these cases, but lower values (e.g. Clark, 1952, 1953a,b) and higher values have been reported for some reptiles. The RE of the eggs of *E. macquarii* was measured for comparison.

Total energy cost of incubation was estimated from total O_2 consumption ($V_{O_2(Tot)}$) and compared to the difference in lipid content between fresh eggs and hatchlings. $V_{O_2(Tot)}$ was measured in eggs at two different temperatures, and therefore two different incubation times, because it is assumed that, for eggs of comparable mass, $V_{O_2(Tot)}$ is smaller if the incubation period is shorter. This is so for reptiles (Ackerman, 1981b) but not birds (Rahn *et al.*, 1974). Incubation time (I) is related to egg weight (W) and maximum O_2 consumption ($\dot{V}_{O_2(max)}$) in avian eggs:

$$I \propto \frac{W}{\dot{V}_{O_2(max)}} \qquad (4.1)$$ (Rahn *et al.*, 1974).

By integrating this equation with respect to incubation time, it was shown that total O_2 consumption is related to egg weight and not incubation time (Rahn *et al.*, 1974). Therefore, if an hypothetical egg takes twice as long to incubate as an egg of equivalent mass, the total O_2 consumed would be the same but the $\dot{V}_{O_2(max)}$ (and conductance) would be halved. In avian eggs differences in incubation period are correlated with differences in eggshell conductance (Rahn *et al.*, 1974). The predicted values for incubation time closely matched the measured values for three pairs of birds with precocial hatchlings and eggs of similar masses but different incubation times and eggshell conductances (Rahn *et al.*, 1974).

Total O_2 consumed during incubation was calculated, on the basis of fresh egg weight in 28 species of birds, at 102 ± 20 $ml.g^{-1}$ (range = 61 - 141 $ml.g^{-1}$) and is independent of incubation time (Hoyt & Rahn, 1980). The O_2 cost during incubation of a reptilian egg is 33 - 40% that of an avian egg with a similar incubation time. This is attributed to the different incubation temperatures of reptilian and avian eggs and indicates that an energetic advantage is gained through development at cooler temperatures (Ackerman, 1981b). The mass-specific O_2 consumption of eggs of *E. macquarii* at two temperatures, and therefore two incubation times, were compared to those of avian eggs of similar masses or incubation times.

A Q_{10} of 2.9, based on the work of Benedict (1932), has been used to adjust the $\dot{V}_{O_2}$ of chelonian eggs incubated at different temperatures to allow interspecific comparisons (Seymour, 1979). Incubation of eggs of *E. macquarii* at two temperatures provided an opportunity for measurement of Q_{10} for comparison.

4.2 MATERIALS AND METHODS

4.2.1 Oxygen Consumption

Oxygen consumption, $\dot{V}_{O_2}$ ($ml.h^{-1}$), of eggs of *E. macquarii*, was measured throughout the incubation period by closed manometry. Early experiments were carried out using 12 single vessel respirometers (Fig. 4.2), immersed in a water bath at 25°C. $\dot{V}_{O_2}$ was measured in 22 eggs from five field-collected clutches. Eleven eggs (from four clutches) were collected at Lake Bonney within 24 hours of being laid and were incubated at 25°C buried in sand from the lake shore (Chapter 2), in a water potential that fluctuated (~ -700 - -2,000 kPa). The remainder had completed part of their development when collected at Lake Alexandrina: their mean $\dot{V}_{O_2}$ was equal to that for day 26 of incubation at 25°C. It was assumed that the subsequent development of those eggs would be at the same rate as the other eggs at 25°C, so the $\dot{V}_{O_2}$ measurements were used to augment the other results from day 26 onwards. The time to hatching indicated that this assumption was valid (Table 4.1). The respiratory chambers took less than an hour to attain thermal equilibrium while open to the atmosphere. The chambers were then closed and the $\dot{V}_{O_2}$ determined from movement of the Eosin indicator drop in the pipette (Fig. 4.2). One chamber containing plasticine of the same volume as an egg was used as a thermobarometer. A KOH solution (25%) was used to absorb CO_2.

Later in the study a Gilson differential respirometer (Fig. 4.3) was used to measure $\dot{V}_{O_2}$ at both 25 and 30°C and NaOH (25%) was used to absorb CO_2. All but two of the eggs were collected from nests at Lake Bonney. These two exceptions were obtained from different females induced to lay in the laboratory (Chapter 2).

$\dot{V}_{O_2}$ of pipped eggs and hatchlings was measured also. On emergence from the eggshell, hatchlings were brushed free of adhering sand, weighed

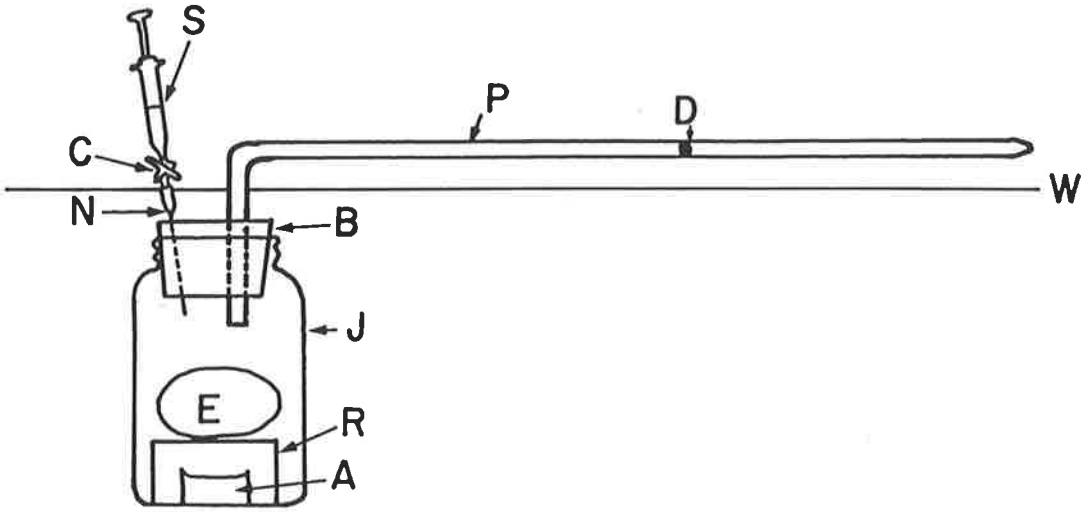


FIGURE 4.2 MANOMETRY CHAMBER USED IN 1979-80.

P = 1 ml pipette coated with silicon, D = eosin indicator drop, W = water level, B = rubber stopper, S = 1 ml syringe, N = needle, C = threeway stop cock, A = CO_2 absorber (25% KOH), R = rack, J = jar, E = egg.

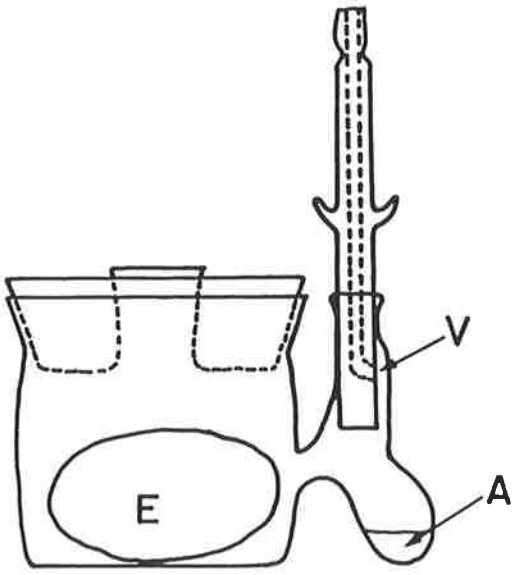


FIGURE 4.3

CLOSED MANOMETER USED WITH GILSON DIFFERENTIAL RESPIROMETER IN 1981-82.

V = vent
A = carbondioxide absorber (25% N_aOH)

and placed in a container of tap water at the same temperature as their incubation. They were not fed. Hatchlings became accustomed to the respiratory chambers during the equilibration time and rested while the measurements were made. $\dot{V}_{O_2}$ was measured daily until they began to search for food; this usually did not occur for several days while they metabolised their remaining yolk reserves.

The total O_2 consumption during incubation ($V_{O_2(Tot)}$ (ml)) was obtained by integrating $\dot{V}_{O_2}$ (STPD) of each egg over the incubation period. This was done by weighing standardized graph paper.

4.2.2 CO_2 Production

CO_2 released during metabolism was determined in Gilson manometry chambers with and without NaOH. $\dot{V}_{CO_2}$ is equal to $\dot{V}_{O_2}$ minus the difference between the two measurements (Grodzinski *et al.*, 1975). RE is:

$$\frac{\dot{V}_{CO_2}}{\dot{V}_{O_2}} \qquad (4.2).$$

All measurements of $\dot{V}_{CO_2}$ were made late in the incubation period because earlier the respiratory rate is so low that small errors in measurement result in a large error in $\dot{V}_{CO_2}$. Ten measurements of RE were made on eggs incubated at 25°C and 26 measurements at 30°C. Not all measurements were made at the same time of the day.

4.2.3 Lipid Measurements

The lipid content of fresh eggs and hatchlings were determined by isolating the lipid in a solution of chloroform after samples were homo-

genised in a 2:2:1 mixture of chloroform, methanol and water (Bligh & Dyer, 1959). Yolk and albumen fractions of fresh eggs were separated for lipid analysis. Once the embryo had adhered to the shell membrane early in the incubation period (Chapter 5) it was difficult to remove the egg contents without rupturing the vitelline membrane. Consequently, the albumen fraction of some eggs was contaminated with a little yolk, but this did not greatly affect the results. Hatchlings from eggs incubated at 30°C were killed by freezing, and these and the yolk and albumen fractions of fresh eggs were dried over silica gel at room temperature under a vacuum to reduce loss of volatile lipid fractions (Ricklefs, 1974). Ten replicates of each were used in the analysis.

4.3 RESULTS

4.3.1 Oxygen Consumption

The pattern of $\dot{V}_{O_2}$ ($ml.h^{-1}$) in all trials was the same. An exponential increase was observed during the first 80% of the incubation period after which the rate of increase of $\dot{V}_{O_2}$ slowed, peaking 87.6 ± 5.5% (n = 13) of the way through the incubation at 30°C and 87.2 ± 5.5% (n = 22) at 25°C. It then declined to 61 - 82% of the peak value before hatching (Figs. 4.4, 4.5).

$\dot{V}_{O_2}$ was measured at 25°C in three separate groups of eggs:

(1) from Lake Bonney, $\dot{V}_{O_2}$ measured in single vessel respirometers,

(2) from Lake Alexandrina, $\dot{V}_{O_2}$ measured in single vessel respirometers,

(3) from Lake Bonney, $\dot{V}_{O_2}$ measured using the Gilson differential respirometer.

Slight differences in incubation times were observed, that for eggs from Lake Alexandrina being estimated assuming 26 days of incubation prior to collection (Table 4.1). Hence comparisons were made on the basis of percent hatching times.

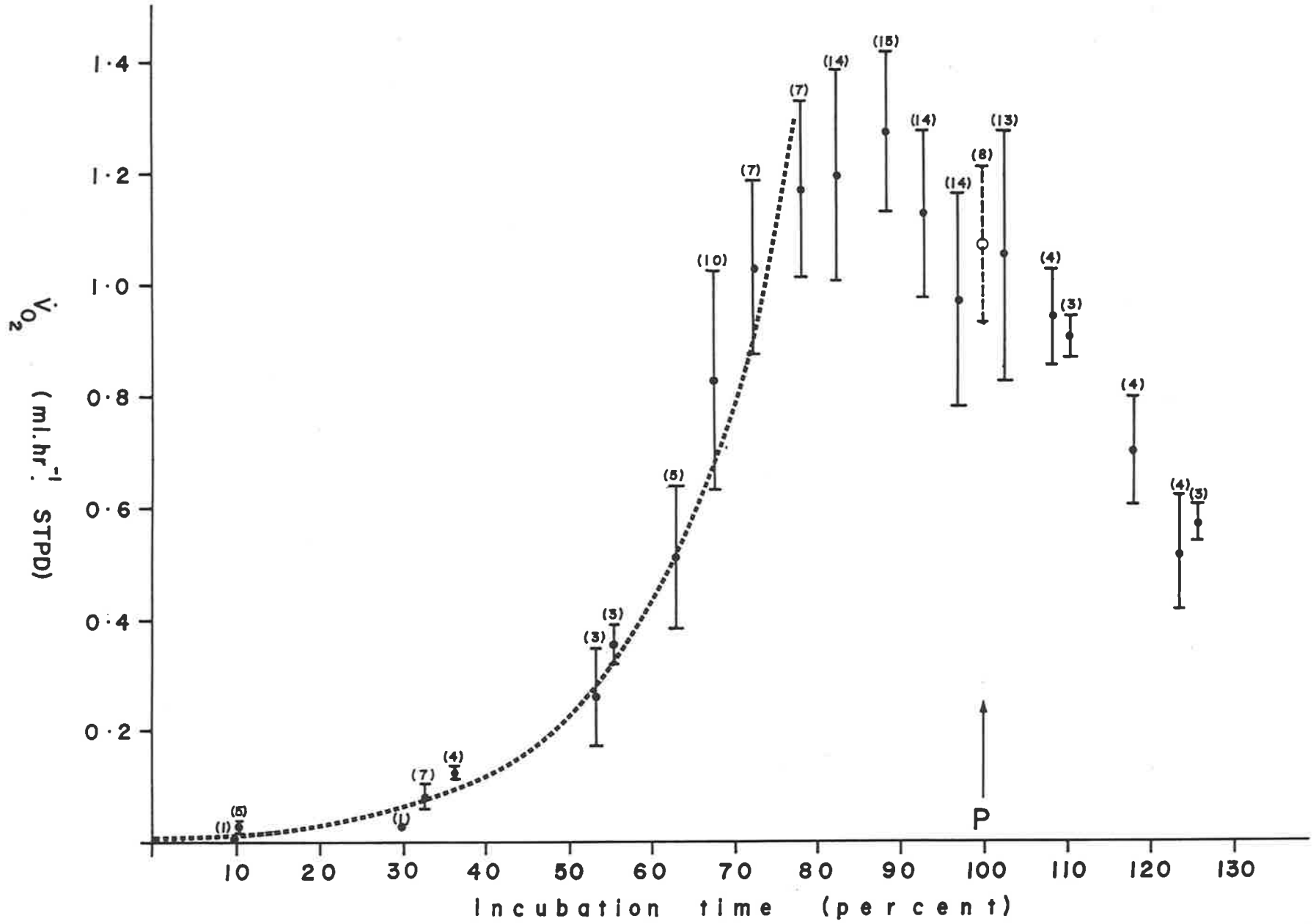
TABLE 4.1

Trial	Time to Pipping	Time to Emergence	n
25°C (1)	60.2 ± 2.7	63.8 ± 0.4	9
25°C (2)	70.7 ± 0.8	71.3 ± 0.8	7
25°C (3)	74.2 ± 2.5	74.9 ± 3.1	9
30°C	47.6 ± 1.7	48.2 ± 1.3	13

Mean time from laying to pipping and laying to emergence from the shell (days) (± S.D.) recorded for eggs used in the determination of $\dot{V}_{O_2}$. Three separate groups of eggs were used at 25°C - see text for explanation.

FIGURE 4.5

Oxygen consumption (STPD) of eggs and hatchlings of *E. macquarii* incubated at $30^{o}C$ expressed as a percentage of incubation time.
Other information as in Fig. 4.3.
Regression line (equation 4.3) is fitted.


1·4
1·2
1·0
0·8
0·6
0·4
0·2
$\dot{V}_{O_2}$ (ml.hr.$^{-1}$ STPD)
10
20
30
40
50
60
70
80
90
100
110
120
130
Incubation time (percent)
P
(1)
(5)
(1)
(7)
(4)
(3)
(3)
(5)
(10)
(7)
(7)
(14)
(15)
(14)
(14)
(8)
(13)
(4)
(3)
(4)
(4)
(3)

An analysis of covariance (Zar, 1974) showed no difference in the linear regressions relating ln $\dot{V}_{O_2}$ to percentage incubation time (I) during the first 80% of the incubation for the different groups of eggs incubated at 25°C ($F_{0.05(2,180)}$ = 3.05, 0.10>p>0.05) so the data were pooled. The linear regression relating ln $\dot{V}_{O_2}$ to percentage incubation time (I) at 30°C during the first 80% of the incubation differed significantly from the pooled result at 25°C ($t_{(2),235}$ = 1,882.4, p<<0.001) (Table 4.2).

At 30°C the relationship was:

$$\ln \dot{V}_{O_2} = 0.0645I - 4.710 \quad (r^2 = 0.932, n = 53) \qquad (4.3)$$

and at 25°C it was:

$$\ln \dot{V}_{O_2} = 0.0560I - 4.601 \quad (r^2 = 0.944, n = 186) \qquad (4.4).$$

A slight increase in $\dot{V}_{O_2}$ was recorded during the pipping of the eggshell but this was not as great as the peak $\dot{V}_{O_2}$ 87% of the way through the incubation. The $\dot{V}_{O_2}$ of hatchlings fell steadily after hatching to levels about half the peak value during incubation (Figs. 4.4, 4.5) before they began to show signs of hunger and measurements were stopped.

Almost equal amounts of O_2 were consumed during incubation prior to pipping at 25 and 30°C (Table 4.2). The amount of O_2 consumed between pipping and emergence from the shell was more variable in eggs incubated at 25°C than at 30°C but the mean values were similar. The $V_{O_2(Tot)}$ for the incubation prior to pipping was divided by the weight of the hatchling to give mass-specific V_{O_2} values of 110.4 ± 13.7 $ml.g^{-1}$ (n = 9) at 25°C and 112.1 ± 14.5 $ml.g^{-1}$ (n = 9) at 30°C.

The embryonic growth rate of *E. macquarii*, as indicated by wet mass including the extra-embryonic membranes (Fig. 4.6), shows a similar pattern to that of $\dot{V}_{O_2}$, increasing exponentially during the first 70% of

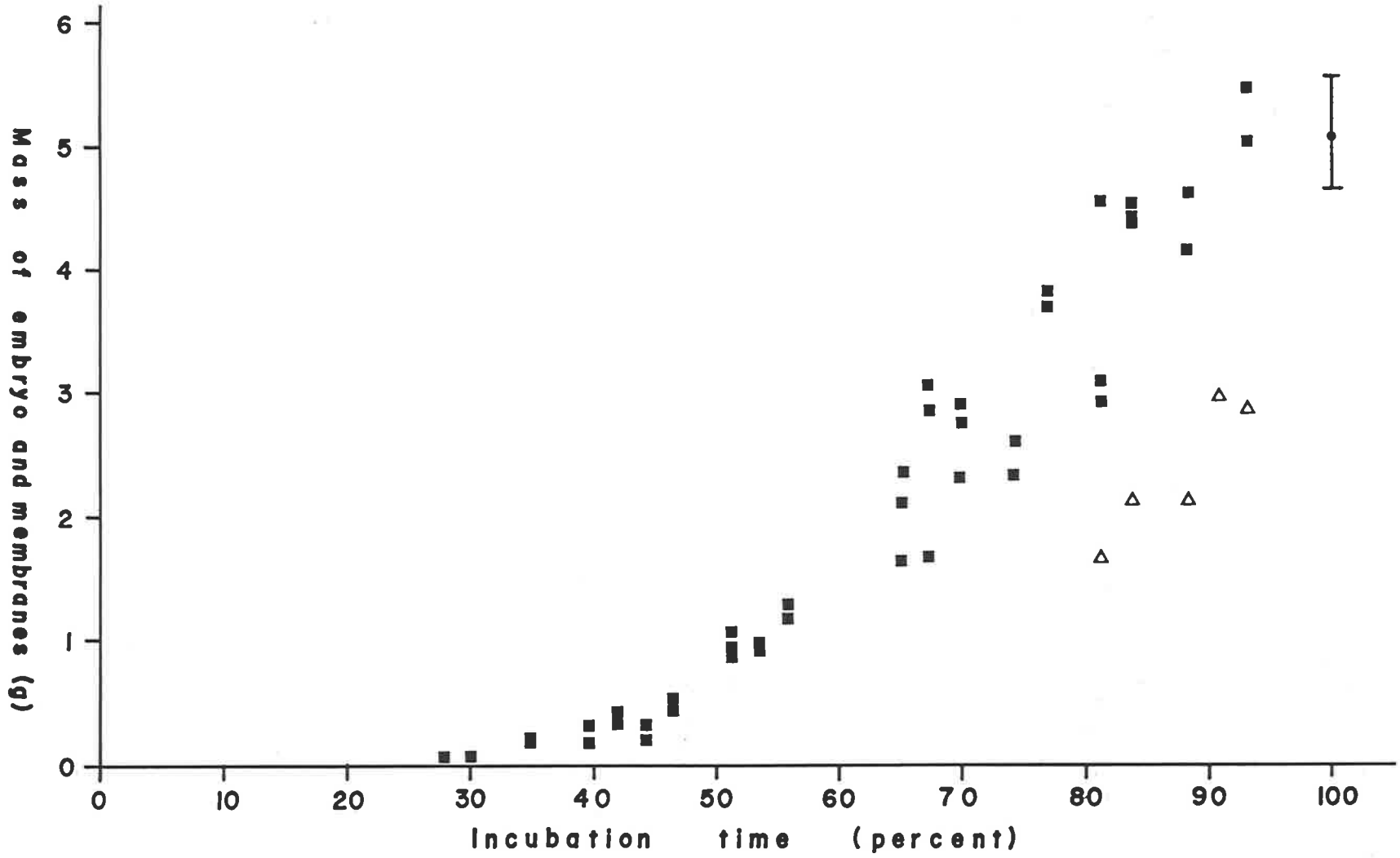


FIGURE 4.6 MASS OF EMBRYONIC *E. MACQUARII* INCLUDING EXTRA-EMBRYONIC MEMBRANES THROUGHOUT INCUBATION AT 30°C. EMBRYOS MARKED WITH Δ WERE RUNTS UNLIKELY TO HATCH. MEAN HATCHLING WEIGHT ± S.D. (n = 110) FROM CHPT. 3 IS GIVEN AT 100% OF THE INCUBATION.

TABLE 4.2 TOTAL O_2 CONSUMPTION, $V_{O_2(Tot)}$ (ml) FOR EGGS INCUBATED AT 25 AND 30°C.

	$V_{O_2(Tot)}$ (Prepipping)				$V_{O_2(Tot)}$ (Pipping-hatch)				TOTAL
Trial	n	$\bar{x}$	S.D.	Range	n	$\bar{x}$	S.D.	Range	
25°C	12	579.7	66.4	439.3 - 704.1	8	59.7	54.1	14.9 - 180.7	639.4
30°C	11	575.8	68.7	461.0 - 685.6	6	49.4	18.4	24.3 - 73.1	625.2

the incubation. The regression equation relating ln mass (W) to percent incubation time (I) was $\ln W = 0.0856I - 4.791$ ($n = 29$, $r^2 = 0.932$). Growth rate slowed after 70% of the incubation.

Mean $\dot{V}_{O_2}$ and corrected mean embryonic mass (W), including the extra-embryonic membranes, at 30°C was calculated for each 5% period of the incubation time. As the fractional water content of embryos decreases during the incubation (Fig. 3.10) the mean embryonic mass was corrected to the water content at hatching (76.95%, Table 3.7) by multiplying the embryonic wet mass by 76.95 and dividing by the water content of an embryo of that age (Fig. 3.10). Data were available for $\dot{V}_{O_2}$ and embryonic mass for eleven of the 5% intervals between 25 and 95% of the way through the incubation and were related by:

$$\ln \dot{V}_{O_2} = 0.8621 \ln W - 0.877 \quad (r^2 = 0.984, n = 11) \qquad (4.5).$$

4.3.2 Respiratory Exchange Ratio (RE)

The mean RE was 0.64 ± 0.05 ($n = 10$) at 25°C and 0.59 ± 0.08 ($n = 26$) at 30°C. The variances and means were equal ($F_{(2)9,25} = 0.331$, $p>0.50$ and $t = 1.450$, $0.20>p>0.10$ respectively) so the data were pooled to give an overall mean of 0.61 ± 0.08 ($n = 36$).

4.3.3 Lipid Measurements

Most lipid (94.2%) found in the fresh eggs of *E. macquarii* is contained in the yolk; the lipid content of hatchlings is 33.8% that of fresh eggs (Table 4.3).

TABLE 4.3 MEAN AND STANDARD DEVIATIONS OF WET AND DRY WEIGHTS OF EGG YOLK, ALBUMEN AND HATCHLINGS
(Ten samples were analysed in each category)

	Fresh Eggs				Hatchling	
	Yolk		Albumen			
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
Wet weight (g)	4.514	1.508	4.425	0.711	4.917	0.307
Dry weight (g)	1.375	0.284	0.128	0.071	1.150	0.097
Lipid (% of dry wt)	24.61	1.55	14.88	7.74	10.70	1.96
Lipid weight (g)	0.340	0.079	0.021	0.016	0.122	0.020

4.3.4 Q_{10} and Maximum O_2 Consumption

The measurements of $\dot{V}_{O_2}$ in two groups of eggs at different temperatures allow the calculation of the Q_{10} with the equation:

$$\text{Log } Q_{10} = \frac{10 \log \dfrac{\dot{V}_{O_2(2)}}{\dot{V}_{O_2(1)}}}{t_2 - t_1} \qquad (4.6)$$

where t_1 and t_2 are the temperatures at which $\dot{V}_{O_2(1)}$ and $\dot{V}_{O_2(2)}$ were recorded. Solving equation 4.6 for estimates of $\dot{V}_{O_2}$ from equations 4.3 and 4.4, 75% of the way through the incubation (0.670 $ml.h^{-1}$ and 1.136 $ml.h^{-1}$) gave a Q_{10} of 2.87. Estimates for this stage of incubation were used because this is the maximum respiration at which equations 4.3 and 4.4 accurately describe the relationship between $\dot{V}_{O_2}$ and incubation time

(Figs. 4.3. & 4.4). At 80% of the incubation the regressions slightly overestimate $\dot{V}_{O_2}$ at both 25 and 30°C.

4.4 DISCUSSION

4.4.1 Patterns of Embryonic O_2 Consumption

The pattern of embryonic O_2 consumption shown in *E. macquarii* (Figs. 4.4, 4.5) is clearly peaked, similar to the condition in ratites. In this respect eggs of *E. macquarii* are similar to those of *Trionyx spiniferus* but different from many other species of chelonians (Lynn & von Brand, 1945; Ackerman, 1981b; Hoyt & Albers, in press). Peaked patterns may be more common than thought earlier. Careful examination of the data of Lynn and von Brand (1945) show the species *Terrapene carolina* and *Chelydra serpentina* have peaked patterns of respiration and not sigmoidal patterns as suggested by the authors.

Those chelonian species with exponential patterns of O_2 consumption during incubation are large, lay large clutches of eggs, live in marine environments and are in the family Cheloniidae (Table 4.4). In contrast, species with sigmoidal patterns of O_2 consumption during incubation are small, live in fresh water and lay few eggs. Species showing the peaked pattern are not separated from those with a sigmoidal pattern on the basis of phylogenetic relationships or habitat preferences, but they tend to be intermediate in body size and clutch size between the other two groups (Table 4.4).

The decline in O_2 consumption after a peak in some birds is a resting stage that allows the less developed embryos to catch up to the more advanced ones to allow synchronous hatching (D. Vleck *et al.*, 1980), i.e. this stage can be shortened to allow synchronous hatching. For example, rhea eggs can be stimulated to hatch naturally at the peak of O_2 consumption by placing them with a group of older eggs (Bruning, 1974). Synchronous hatching was found in two species of quail by either a combination of retarding advanced and accelerating early embryos or by acceleration

TABLE 4.4 REPRODUCTIVE DATA ON SPECIES FOR WHICH O_2 CONSUMPTION HAS BEEN MEASURED THROUGHOUT INCUBATION

Species	Family	Habitat	♀ Carapace Length(cm)	No. of Egg / Clutch	Fresh Egg Wt(g)	Hatch Wt(g)
Exponential Pattern						
Caretta caretta[1]	Cheloniidae	Marine	100[9]	100 - 140[6]	34.5[7]	16.0[7]
Chelonia mydas[1]	Cheloniidae	Marine	90 - 130[5]	50 - 200[5]	51.6[7]	25.1[7]
Sigmoidal Pattern						
Chrysemys concinna[2]	Emydidae	Freshwater	28[8]		12.2[2]	8.3[2]
Chrysemys picta[3]	Emydidae	Freshwater	13 - 16[3]	2 - 8[3]	5.3[2]	4.3[2]
Kinosternon subrubrum[3]	Kinosternidae	Freshwater	8 - 12[8]	2 - 3[3]	4.2[2]	3.1[2]
Peaked Pattern						
Emydura macquarii[4]	Chelidae	Freshwater	30[7]	18 - 33[4]	10.4[4]	~ 5
Trionyx spiniferus[2]	Trionychidae	Freshwater	<40[8]	10 - 25[8]	9.1[2]	6.6[2]
Terrapena carolina[3]	Emydidae	Terrestrial	<18[7]	2 - 3[3]	9.7[2]	7.1[2]
Chelydra serpentina[3]	Chelydridae	Freshwater	27 - 33[3]	29 - 61[3]	9.9[2]	7.2[2]

References:

1. Ackerman, 1981b
2. Hoyt & Albers, in press
3. Lynn & von Brand, 1945
4. This study
5. Bustard, 1972
6. Ackerman, 1977
7. Ewert, 1979
8. Carr, 1952
9. Cogger, 1979

alone (Vince, 1964, 1968), as suggested in the rhea.

4.4.2 Adaptive Value of Developmental Patterns of O_2 Consumption

The different patterns of embryonic O_2 consumption in the Chelonia may be explained in the same way as birds. Simultaneous hatching may be important in chelonian nests to enable hatchlings to dig out of the nest chamber (Hendrickson, 1958; Carr & Hirth, 1961). Simultaneous emergence may also be a strategy to 'swamp' predators, allowing a greater survival of hatchlings (Carr, 1967). All eggs in a chelonian clutch are laid at the same time and stage so differences in incubation time within a clutch result from different developmental rates, which are related to the incubation temperature. Nest depth is generally positively correlated with female size because of the stereotyped way that nests are constructed in the Chelonia (Carr, 1967; Mahmoud, 1968; Ernst, 1970). Hence, large species (e.g. marine turtles) construct deep nests and small species construct shallow ones. The nests of the marine turtles whose pattern of embryonic O_2 consumption is known (*Caretta caretta* and *Chelonia mydas*) are buried deeply enough not to be influenced by diurnal temperature fluctuations (Hendrickson, 1958; Carr & Hirth, 1961; Bustard, 1972). In such a thermally stable environment all eggs are likely to develop at essentially the same rate and therefore to hatch at the same time. There may be some temperature difference between the eggs in the centre of the nest and those adjacent to the wall of the nest chamber due to metabolic heating (Bustard, 1972). However, by the time a significant gradient develops, the rate of growth would be independent of temperature (Yntema, 1968, 1978).

In contrast, clutches of intermediate size (e.g. *E. macquarii*, Table 4.4), incubated in shallow nests, are influenced by daily temperature

fluctuations (Burger, 1976) such that the top eggs experience warmer temperatures overall than the bottom eggs (Fig. 7.7). This causes considerable variation in developmental rates, and therefore hatching times (Goode, 1967). If synchronous hatching is advantageous then a 'catch-up period' (D. Vleck *et al.*, 1980) would be expected in these species. Small clutches (2 - 8) probably consist of one layer of eggs in the nest and so will experience essentially the same temperature regime. The catch-up period will probably be short, if necessary at all, and the pattern of embryonic O_2 consumption sigmoidal, resembling a truncated version of the peaked pattern observed in clutches of intermediate size where a longer catch-up period is advantageous.

Species with an exponential pattern of O_2 consumption are exclusively marine and more closely related to each other than to species showing sigmoidal or peaked patterns. However, the explanation for the different patterns of embryonic O_2 consumption assumes that the temperature regime experienced by the clutch is more important than the phylogenetic relationships and habitat preferences of the species. This could easily be tested by observing three species.

1. The leatherback, *Dermochelys coriacea*, is a marine turtle which lays large clutches of eggs deeply buried in the sand (Prange & Ackerman, 1974). It belongs to a different family (Dermochelydae) from the other marine turtles and has sometimes been placed in a different suborder (Pritchard, 1971). The hypothesis predicts that this species should show a typical exponential pattern of embryonic O_2 consumption.

2. Because nest depth is positively correlated with female size (Carr, 1967; Mahmoud, 1968; Ernst, 1970) a small marine turtle in the Cheloniidae, such as the olive ridley (*Lepidochelys olivacea*) (Bustard, 1972; Ackerman, 1980) is likely to lay eggs in shallower

nests than other marine turtles. As such, the eggs should experience some diurnal temperature influence and the embryos should show a peaked pattern of O_2 consumption.

3. For similar reasons, a large freshwater species, such as the pig-nosed turtle (*Carettochelys insculpta*), is likely to construct deep nests. The eggs of this species should therefore show an exponential pattern of embryonic O_2 consumption.

The hypothesis itself could be tested by placing some less advanced eggs in a clutch of advanced eggs. It predicts synchronous hatching, as in the rhea (D. Vleck *et al.*, 1980) and quail (Vince, 1968). The actual stimulus for pipping the shell in *E. macquarii* is not known. Although auditory stimulation has been shown to accelerate hatching in the eggs of Japanese quail (Woolf *et al.*, 1976), vocalisation is unlikely in chelonians (Bustard, 1972); no *E. macquarii* were heard to vocalise. In marine turtles it has been shown that the movement of a hatchling is the stimulus for the other eggs to hatch (Bustard, 1972). As eggs of *E. macquarii* were incubated in such a way that they did not contact each other this process could not have applied in the experiments and so the eggs would not have been stimulated to hatch early. Those eggs that hatched after a peak 98% of the way through the incubation may have been stimulated to emerge early by movement associated with placing them in the respiratory chambers, but this is not substantiated.

Peak $\dot{V}_{O_2}$ occurred 87% of the way through the incubation at both 25 and 30°C even though the mean incubation times differed (65 days at 25°C and 47 days at 30°C: Fig. 7.8). This gives catch-up times of 8.3 and 5.8 days at 25 and 30°C respectively. As incubation time is inversely related to incubation temperature (Chapter 7), a temperature difference between the top and bottom eggs in a clutch will cause a greater difference in incuba-

tion time between these eggs in nests with a lower mean incubation temperature than the same temperature difference in a nest incubated at a higher mean temperature. Similarly, a temperature difference between the top and the bottom eggs early in the incubation will cause a greater difference in incubation time than the same temperature difference experienced later because the rate of embryonic development becomes less dependent on temperature as the incubation proceeds (Yntema, 1978). No information is available for *E. macquarii* on the rate of embryonic growth at different temperatures, i.e. on how it varies throughout the incubation and how the incubation period is altered from that at constant temperature if the temperature fluctuates about a mean equal to the constant temperature. Without this information, and because of the wide daily and seasonal fluctuations of temperature experienced by a nest of *E. macquarii*, calculations to determine the difference in hatching time in the field are not possible.

The logarithm of $\dot{V}_{O_2}$ is linearly related to the logarithm of embryonic mass with a slope of 0.862 (i.e. $\dot{V}_{O_2}$ is related to embryonic mass raised to the power 0.862; equation 4.5). Similar relationships have been reported for embryos of snakes (Dmi'el, 1970) and birds (C. Vleck *et al.*, 1979). The exponent relating $\dot{V}_{O_2}$ to body mass for animals is generally about 0.75 (Bartholomew, 1972). The mean value of 0.92 obtained for avian eggs (C. Vleck *et al.*, 1979) is similar to that obtained for the eggs of *E. macquarii*, and more than that predicted from the general relationship. This increase was shown theoretically to be due to the higher energy cost of biosynthesis, or rapid growth, in avian embryos (C. Vleck *et al.*, 1979), and may also explain the value obtained for the eggs of *E. macquarii*.

4.4.3 Q_{10} and Maximum O_2 Consumption

The calculated Q_{10} of 2.87 for eggs of *E. macquarii* compares favourably with the value of 2.9 that has been used to adjust the $\dot{V}_{O_2}$ of chelonian eggs to allow interspecific comparisons (Seymour, 1979).

Maximum $\dot{V}_{O_2}$ is related to egg weight (W, g) at 30°C for reptiles:

$$\dot{V}_{O_2\,(max)} = 0.244.\ W^{0.737}\ ml.h^{-1} \qquad (4.7)$$ (Seymour, 1979).

Using 10.423 g, the mean fresh egg weight of *E. macquarii* (Chapter 3), the predicted value of $\dot{V}_{O_2\,(max)}$ at 30°C is 1.37 $ml.h^{-1}$, close to the $\dot{V}_{O_2\,(max)}$ measured at 30°C (1.28 $ml.h^{-1}$) (Fig. 4.5).

Similar equations have been developed for avian eggs at 38°C (Rahn *et al.*, 1974; Hoyt & Rahn, 1980). Rates of O_2 consumption by eggs of *E. macquarii* at 38°C calculated using a Q_{10} of 2.87 fall short of the values estimated from equations for avian eggs by 37 - 50%, supporting the idea that energetic cost of incubation is less in reptiles than birds.

4.4.4 Total Embryonic O_2 Consumption

The eggs of *E. macquarii* showed a greater $\dot{V}_{O_2\,(max)}$ with more rapid growth at 30°C than at 25°C (Figs. 4.3, 4.4) but there was no significant difference between the total O_2 consumed at these two temperatures (Table 4.2). This is in contrast to the results for species in which the total amount of O_2 consumed increased with incubation time (Ackerman, 1981b). However, total O_2 consumption is not increased with incubation time in many avian eggs (Ar & Rahn, 1978; Hoyt & Rahn, 1980).

The energetic cost of embryonic development consists of the cost of growth plus the cost of maintenance (C. Vleck *et al.*, 1980). Consequently,

in eggs of equal mass, any increase in energetic cost with increased incubation time must come from increased maintenance. Since there is no increase in the energetic cost in eggs of *E. macquarii* with a difference in incubation time of more than 40% (Table 4.1) then the cost of maintenance must also rise with increased temperature.

According to equation 4.1, incubation time is inversely related to $\dot{V}_{O_2(max)}$. Therefore, as the ratio of incubation times (0.70) (from Fig. 7.8) is very close to the inverse of the ratio of $\dot{V}_{O_2(max)}$ (0.72) for eggs of *E. macquarii* incubated at 25 and 30°C increased incubation time does not incur greater energetic expense.

Mass specific total O_2 consumptions of 55.6 and 55.2 $ml.g^{-1}$ for the incubation at 25 and 30°C respectively were calculated for *E. macquarii* by dividing the O_2 consumption during the incubation (Table 4.2) by the mean weight of fresh eggs (10.423 g, Chapter 3). These values are lower than any obtained by Hoyt and Rahn (1980) (61 - 141 $ml.g^{-1}$) in 28 species of birds with eggs ranging in size from 1.0 - 1,450 g and incubation times ranging from 12 - 79 days. They are about 45% lower than the value obtained for quail, *Coturnix coturnix* (which has a similar sized egg to that of *E. macquarii*), and 15 - 55% lower than species with incubation periods greater than 40 days. As the hatchling sizes are similar, this indicates that less energy is required to produce 1g of *E. macquarii* than 1g of avian tissue and correlates with the estimate that the O_2 cost of the reptilian egg is only 33 - 40% that of the avian egg (Ackerman, 1981b). This difference has been attributed to the different incubation temperatures of reptilian and avian eggs where the Q_{10} of the metabolic process underlying growth exceeds 1; this is true for *E. macquarii* (see 4.4.2), and supports the idea that there is an energetic advantage in incubation at cooler temperatures (Ackerman, 1981b).

4.4.5 Hatchling O_2 Consumption

After emerging from the eggshell the $\dot{V}_{O_2}$ of hatchling *E. macquarii* was observed to fall gradually until measurements were stopped (Figs. 4.3, 4.4). Falls in $\dot{V}_{O_2}$ after hatching have been reported in the chelonians *Chelydra serpentina* (Lynn & von Brand, 1945) and *Chelonia mydas* (Prange & Ackerman, 1974) and the snake, *Liopeltis vernalis* (Zarrow & Pomerat, 1937). However, two other species of chelonian, *Chrysemys picta* and *Kinosternon subrurum*, showed a rise in O_2 consumption in the first 8 and 14 days after hatching respectively (Lynn & von Brand, 1945). The $\dot{V}_{O_2}$ of hatchling birds rises steeply after hatching because they are still growing rapidly and, as endotherms, they thermoregulate (C. Vleck *et al.*, 1979).

The prehatching metabolic rate found in *E. macquarii* is higher than the metabolic rate of hatchlings (Figs. 4.4, 4.5), which supports the finding that the prehatching metabolic rate of reptilian embryos is higher (20%) than the metabolic rate of adult reptiles of equal body mass (Hoyt & Albers, in press). The rate of O_2 consumption of avian embryos is correlated with both embryonic mass and rate of increase of embryonic mass; these components account for the energy cost of basal metabolism and growth respectively (C. Vleck *et al.*, 1980). Hence the plateau in embryonic O_2 consumption in species with sigmoidal patterns represents a slight decline in growth rate, the peaked pattern a large decline and the exponential pattern a continuous growth rate (Hoyt & Albers, in press). In support of this conclusion the rate of embryonic growth in *E. macquarii* declines towards the end of the incubation (Fig. 4.6). Further, the decline in metabolism of hatchling *E. macquarii* indicates a continued decline in the rate of growth after hatching.

The slight rise in $\dot{V}_{O_2}$ at pipping in *E. macquarii* (Figs. 4.4, 4.5)

reflects the extra energy required for the muscular movement needed to free the hatchling from the egg. The rise associated with hatching is three times the prepipping $\dot{V}_{O_2}$ in *Chelonia mydas* (Ackerman, 1980) and may have included $\dot{V}_{O_2}$ of digging out of the nest chamber.

4.4.6 Respiratory Exchange Ratio

The RE measured in eggs of *E. macquarii* towards the end of incubation (0.61 ± 0.08, n = 36) is lower than that expected if lipid is the sole respiratory substrate. I am unable to explain the low values. Clark (1953b) outlined several theoretical ways in which RE could be depressed. One of these (viz. the conversion of fat to carbohydrate which uses O_2 without releasing CO_2) is supported by the data of von Brand and Lynn (1947). They found 20 mg more carbohydrate in the hatchlings of *Chelydra serpentina* than in fresh eggs. No analysis of change in carbohydrate levels was made for the eggs of *E. macquarii*. Other reasons for low RE may include storage of CO_2 in the blood and tissues, which contain high levels of CO_2 in chelonians (Burggren & Shelton, 1979), and excretion of bicarbonate in solution as in *Crocodylus porosus* (Grigg, 1978).

RE values varied widely in *E. macquarii* eggs, as indicated by the magnitude of the standard deviation. Such wide variations are common in measurements of RE: $\bar{x}$ = 0.71, range = 0.615 - 0.856 in nine species of birds (Rahn *et al.*, 1974); in the chicken $\bar{x}$ = 0.66, range = 0.60 - 1.00 (Barott, 1937) and $\bar{x}$ = 0.71, range = 0.67 - 0.89 (Romijn & Lockhorst, 1955). Variations in measurements of RE in reptilian eggs are even greater than avian eggs, and many authors have reported mean values lower than 0.7. Measurements of RE range from 0.56 - 0.61 in the snake *Elaphe laeta* (Clark, 1952) and from 0.52 - 0.61 in *Elaphe emoryi* (Clark, 1953a). Although both reports obviously refer to the same data (which contain

typographical and identification errors), the main point is the low values of RE. The mean RE for the snake *Natrix natrix* for the last two thirds of the incubation is 0.53 (Clark, 1953b). RE in the eggs of the lizard *Crotaphytus collaris* ranges from 0.513 - 1.07 ($\bar{x}$ = 0.793 ± 0.217, n = 6) (Clark, 1946).

Oxygen consumption was found to undergo a marked circadian rhythm in the eggs of five species of snakes (Dmi'el, 1969) and domestic chickens (Johnson, 1966). If this is a common phenomenon it could explain some of the variation in measurements of RE because the $\dot{V}_{O_2}$ may have changed between the time $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were measured.

RE appeared to be independent of temperature in eggs of *E. macquarii* because the measurements at 25°C fell completely within the range obtained at 30°C. RE was also found to be independent of temperature in the late stages of incubation (the only available data) in the snake, *Liopeltis vernalis* (Zarrow & Pomerat, 1937).

4.4.7 Energy Budget

The importance of lipid as a respiratory substrate during incubation was estimated by calculating the cost of incubation from $V_{O_2(Tot)}$ and measuring the amount of lipid used during incubation. The mean cost of incubation, 12,380 J, was calculated by multiplying the total O_2 consumed during incubation and pipping (Table 4.2) by 19.8, which is the energetic equivalent of 1ml of O_2 (Bartholomew, 1972). Of this 9,500 J, or 76.7%, came from the metabolism of lipid (0.239 g of lipid multiplied by 39,750 J, the energetic equivalent of 1g of lipid, Bartholomew, 1972). The other source of energy was probably protein (Lynn & von Brand, 1945; von Brand & Lynn, 1947; Ewert, 1979). Although no direct measurements were made of

protein utilization in *E. macquarii*, these results indicate that relatively less protein was used during development in *E. macquarii* than in *Chelydra serpentina*, where lipid accounted for 62% of the energy requirements (von Brand & Lynn, 1947).

5

GAS RELATIONS

5.1 INTRODUCTION

The respiratory gases of chelonian, crocodilian, avian and some other embryos must diffuse through the eggshell, outer and inner shell membranes and extraembryonic membranes (chorion and chorioallantois) (Rahn *et al.*, 1979). Convective gas exchange is largely prevented by the rigid shell and small pores (Rahn *et al.*, 1971, 1979; Wangensteen, 1972; Paganelli, 1980).

In avian eggs an airspace forms during development between the inner and outer shell membranes at the blunt end of the egg. Because the shell is the main barrier to diffusion (Bartels, 1970; Wangensteen *et al.*, 1970/71), the airspace is convenient for sampling the internal gases of the egg. Gas tensions in the airspace were assumed to be similar to those at the chorioallantoic membrane and have been measured in the eggs of many birds (Rahn *et al.*, 1974; D. Vleck *et al.*, 1980). During the later part of the incubation period the permeability of the eggshell to gases remains constant (Tullett & Board, 1976), but embryonic respiration increases, causing elevated CO_2 tensions and depressed O_2 tensions in the airspace. The P_{CO_2} levels reach about 40 torr and P_{O_2} about 100 torr in the airspace just prior to hatching (Rahn *et al.*, 1974). In the chicken the final gas concentrations in the airspace are very close to those in the adult lung (Tazawa *et al.*, 1971); this may be an adaptation to enable a smooth transition from chorioallantoic to pulmonary respiration (Wangensteen, 1972). Developing chicks will pip earlier if the airspace P_{CO_2} is raised and P_{O_2} lowered, and pip later if the reverse is true (Visschedijk, 1968b).

Changes in gas tensions in the nests of the marine turtles *Chelonia mydas* and *Caretta caretta* are similar to those in the air cell of a single avian egg (Ackerman, 1977). The ΔP_{CO_2} and ΔP_{O_2} across the eggshell

are small (Prange & Ackerman, 1974), because the conductance is high (Ackerman & Prange, 1972), so the gas tensions experienced by the embryos are close to those of the nest. Sand around the nest restricts gas exchange with the atmosphere (Ackerman, 1977) because movement of gas through soil occurs primarily by diffusion (Hillel, 1973). Ackerman (1977) drew an analogy between a complete turtle's nest and a single avian egg: the sand is functionally similar to the shell of the avian egg and the young turtles act like a single developing avian embryo. He continued (p.34):

> *"it is tempting to suggest that the presence of similar gas partial pressure differences during the embryonic development of two rather dissimilar groups of vertebrates is not fortuitous and serves a physiological function important to both reptilian and avian embryos."*

This implies that the changes in gas tensions in turtle nests and in the air cell of the avian egg are conservative and of fundamental physiological importance. These changes may stimulate pipping and enable a smooth transition between chorioallantoic and pulmonary respiration (Wangensteen, 1972).

To test Ackerman's ideas I determined the gas concentrations experienced by the embryos of *E. macquarii*. I examined three barriers to the exchange of O_2 and CO_2 between the developing embryos and the air:

(1) the 5-8 cm of soil separating the nest cavity from the air,

(2) the calcareous shell of the egg, and

(3) the underlying shell membranes.

These barriers attain their maximum significance when the respiration of the embryo is highest (i.e. 80 - 90% through the incubation; see Fig. 4.7).

It appeared that the conditions in the nests of *E. macquarii* would be

less severe because the clutches are relatively small (200 - 350 g) and shallowly buried (5 - 8 cm). The clutches of marine turtles studied by Ackerman were large (4 - 5 kg) and deeply buried (40 cm), resulting in large partial pressure differences of O_2 and CO_2 between the nest atmosphere and the air.

After laying, the eggs of *E. macquarii* develop an opaque white patch; this enlarges to completely cover some eggs by the time of hatching. A similar phenomenon has been reported in other reptiles (Einem, 1956; Ewert, 1979; Ferguson, 1982). Development of the white patch may be reduced if the outside of the eggshell is in contact with moist material (Ewert, 1979). Shell removed from fresh eggs and dried becomes opaque and white. Translucent patches that contain more water than surrounding opaque shell have been observed in chicken eggs (Tyler, 1969). I investigated the hypothesis that the white patch is an area of the eggshell that dries out to facilitate gas exchange to the developing embryo.

5.2 MATERIALS AND METHODS

5.2.1 Gas Tensions in Nests in the Field

Tubes for sampling gas were implanted into nests at Lake Bonney and at a nearby commercial tortoise farm within 24 hours of construction during two nesting seasons (1978-9; 1979-80). The farm nests were needed to increase the sample size, because intact natural nests were difficult to find (Chapter 8). Nests at the lake were covered with wire mesh to protect them from predators.

The gas sampling tubes, made of polyethylene (I.D. = 0.584 mm, O.D. = 0.965 mm) supported by thick glass tubing (I.D. = 1.0 mm, O.D. = 5.0 mm), were inserted around the nests (Fig. 5.1). At least two layers of plankton netting were attached, with a rubber band, to the buried end so that soil particles were excluded from the tube. At the other end, a 23 lc hypodermic needle was inserted into the polyethylene tubing and fixed to the glass with epoxy resin. The syringe needles were stoppered with plastic plugs to prevent entry of gas, grit and water. The tubes were inserted from a trench dug on one side of the nest without disruption to the nest plug. Eggs were removed through the trench to enable insertion of a tube into the centre of the clutch, and returned to their original positions.

Control samples were taken from sampling tubes buried at depths equivalent to the centre of nests at both the Lake and the tortoise farm in 1978-79 and from an artificial nest containing plastic eggs at the lake in 1979-80.

Gas samples of 1-2 ml were taken in greased glass syringes after expelling at least twice the dead space from each sampling tube, weekly in 1978-9 and intervals of 6-14 days in 1979-80. The syringes were

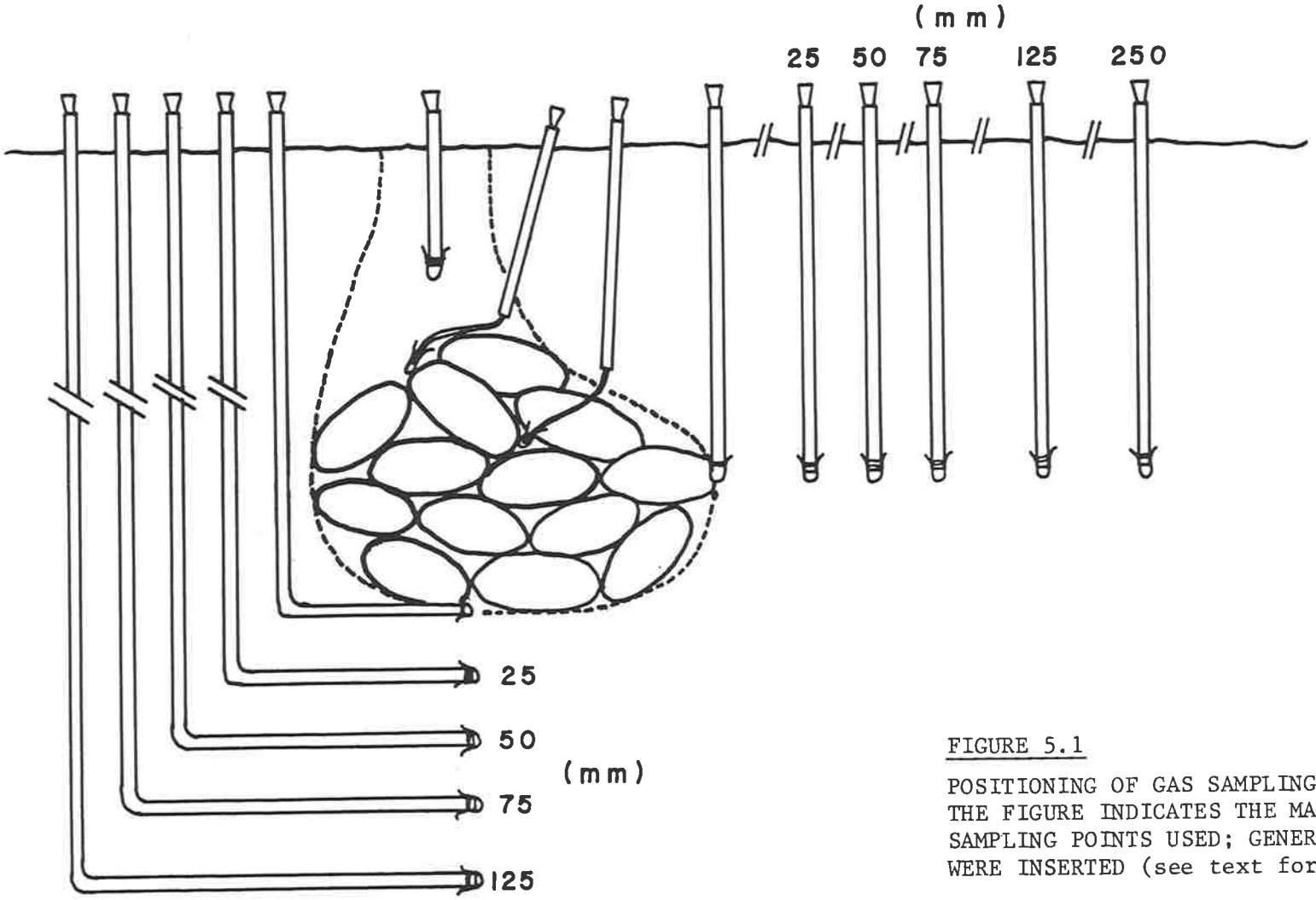


FIGURE 5.1

POSITIONING OF GAS SAMPLING TUBES ABOUT NEST. THE FIGURE INDICATES THE MAXIMUM NUMBER OF SAMPLING POINTS USED; GENERALLY FEWER TUBES WERE INSERTED (see text for description).

sealed with stainless steel caps and stored under water for protection and to enable detection of leaks.

The samples were analysed for O_2 and CO_2 content with a Scholander 0.5 cc gas analyser (Scholander, 1947) in 1978-9 and the beginning of the 1979-80 season, and with O_2 and CO_2 electrodes (Radiometer, Copenhagen) calibrated with precision gas mixtures for the remainder of the second season. For calculation of P_{O_2} and P_{CO_2} the samples were assumed to be saturated with water vapour.

5.2.2 Water Vapour Conductance of the Shell ($G_{H_2O(s)}$*)

Loss of weight from avian eggs is attributed to diffusion of water vapour (Romanoff & Romanoff, 1949; Paganelli *et al.*, 1971; Rahn & Ar, 1974) through gas-filled pores (Paganelli *et al.*, 1975). The conductance of eggshell to water vapour is directly related to its conductance to O_2 and CO_2, according to the laws of gas diffusion (Paganelli *et al.*, 1971; Ar *et al.*, 1974). Conductance ($G_{H_2O(s)}$) was determined by measuring the weight loss of eggs over silica gel at 25°C (Ar *et al.*, 1974).

Strictly, the SI system of units requires conductance to be expressed in $cm^3.day^{-1}.kPa^{-1}$. However, by convention, respiratory physiologists have used $cm^3.day^{-1}.torr^{-1}$ and all conductance values reported in the literature are so expressed. For ease of comparison I have used $cm^3.day^{-1}.torr^{-1}$ (1torr = 1mm Hg = 0.133kPa).

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* Eggshell conductances calculated from water loss data, and therefore referring to the shell alone, are subscripted (s) (thus $G_{H_2O(s)}$) to distinguish them from conductances measured directly in isolated shell with intact membranes; these are subscripted (ms) (thus $G_{O_2(ms)}$).

Conductance of the shell to water vapour was calculated using -

$$Gg = \frac{\dot{M}g}{\Delta Pg} \qquad (5.1) \quad \text{(Ar } et\ al.\text{, 1974)}$$

where Gg = conductance of the diffusion barrier (shell or shell plus membranes) to gas, g, ($cm^3.day^{-1}.torr^{-1}$)

$\dot{M}g$ = the rate of movement of gas, g, across the barrier ($cm^3.day^{-1}$)

ΔPg = partial pressure difference of gas, g, across the barrier (torr).

$G_{O_2(s)}$ and $G_{CO_2(s)}$ were calculated by adjusting the value of $G_{H_2O(s)}$ with the relative diffusion rates of H_2O, O_2 and CO_2 (Paganelli *et al.*, 1978).

Eggs used for these conductance measurements were half buried in substrates with a water potential of about -780kPa and incubated at 25°C. This substrate was chosen because other experiments suggested that there would be minimal net weight change due to water movements under such conditions (Chapter 6). At 25°C eggs were expected to hatch in 60 - 70 days (see Chapter 7), so samples of eggs were placed over silica gel in desiccators after 1, 15, 30, 45 and 57 days of incubation (Table 5.1).

TABLE 5.1 NUMBER OF EGGS FROM EACH CLUTCH USED IN WATER VAPOUR CONDUCTANCE EXPERIMENTS

	No. of Eggs From Each Clutch						
Clutch No.	46	47	49	56	58	61	Total
Trial - Day 1			2	2	1	1	6
Day 15	1	3					4
Day 30	1	3					4
Day 45	2	3					5
Day 57		1	5*				6
Total Fertile	4	10	7	2	1	1	25
Day 57 Infertile	1		1	1		4	7

* One of these eggs hatched after nine days in the desiccator

Infertile and dead eggs were also placed in desiccators after 57 days in the incubation chambers. Table 5.1 indicates the number of eggs used in each trial. All were weighed regularly until they reached constant weight or until they collapsed.

5.2.3 O_2 and CO_2 Conductance of Shell and Membranes ($G_{O_2(ms)}$ and $G_{CO_2(ms)}$)

Direct measurements of the conductance of the shell and membranes to O_2 and CO_2 were made on four clutches and three of these four clutches, respectively.

Before the egg contents were removed for conductance measurements, the white patch was outlined with an HB graphite pencil and a 3 mm hole drilled in each end of the egg. For young eggs the yolk was removed into a syringe and the albumen and embryo expelled from one hole by blowing into the other. Any extra-embryonic membranes adhering to the shell membrane were removed by scraping with a large needle. Eggs containing embryos larger than 3 mm were cut through the equatorial plane with an edge-cutting disc in a dental drill. The contents were removed and the two halves rejoined with Cyanobond RS 100 ® as quickly as possible. Fifty millimeters of polyvynylchloride tubing (I.D. = 2.00 mm, O.D. = 3.00 mm) was glued in each hole in the egg with Cyanobond. These manipulations were conducted in a humidified chamber at 30°C to prevent water loss from the shell and membranes.

The method of measuring conductance is shown in Figure 5.2. The chamber containing the egg was an acrylic cylinder sealed at each end. Four 19 1c hypodermic needles, each fitted with a three-way stopcock, passed through the rubber stopper in the top of the chamber. Two needles

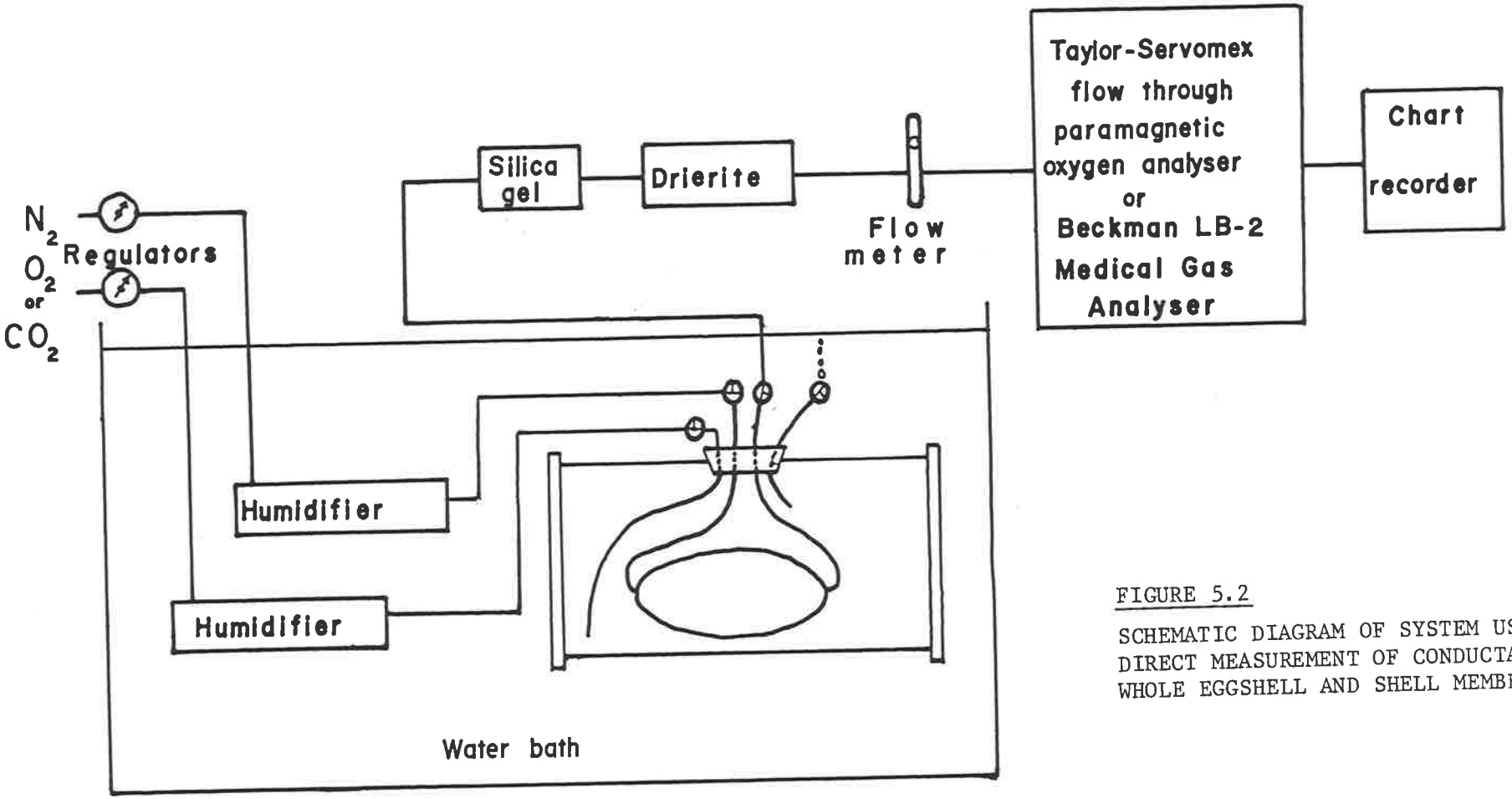


FIGURE 5.2

SCHEMATIC DIAGRAM OF SYSTEM USED IN DIRECT MEASUREMENT OF CONDUCTANCE OF WHOLE EGGSHELL AND SHELL MEMBRANES.

had thin PVC tubing (I.D. = 1.00 mm, O.D. = 2.00 mm) which fitted exactly into the tubing attached to the egg. Tubing connected to the third needle delivered O_2 or CO_2 to one end of the chamber, and the fourth needle vented the chamber to the outside. The whole apparatus was submerged in a water bath at $30^{o}C$. Humidified N_2 was passed through the egg at 325 ml. min^{-1} throughout each experiment. The chamber was flushed with humidified O_2 or CO_2 until equilibrium was reached. Then it was closed via the stopcocks. This allowed pressure differences between the chamber and the egg to equilibrate so that all gas movements would be attributable to diffusion. The concentration of O_2 in the flow of N_2 was determined with a Taylor Servomex flow-through Paramagnetic Oxygen Analyser and CO_2 with a Beckman LB - 2 Medical Gas Analyser each connected to a Perkin-Elmer 56 chart recorder. Because the chamber was large compared to the egg, dilution of O_2 or CO_2 in the chamber by N_2 from the egg was insignificant. At the end of each experiment the egg with N_2 flowing through it was immersed in water to test for leaks around the joints.

Conductance to O_2 and CO_2 was measured on eggs of different age throughout the incubation period. In addition, $G_{O_2(ms)}$ was remeasured in four eggshells and membranes that were dried at room temperature over silica gel.

In the first 10 days of incubation the white patch was outlined on 53 eggs with an HB graphite pencil. These patches, and those outlined prior to measurements of conductance, were traced with wet ink, the eggs rolled on graph paper and the squares counted. The area of the white patch was determined by calculating the mean of three measurements. The mean error, estimated from triplicate measurements, was 4.2%. Egg surface area estimates are described elsewhere (Section 3.2.6).

The water content of translucent and opaque shell from six eggs was determined by drying them to constant weight over silica gel at room temperature.

5.3 RESULTS

5.3.1 Gas Tensions in Nests in the Field

The patterns of change of P_{O_2} and P_{CO_2} in the nests of *E. macquarii* (Figs. 5.3, 5.4) were similar to those for marine turtles (Ackerman, 1977), but the final gas tensions were less severe. P_{O_2} declined in the centre of nests throughout the incubation to about 10 torr below atmospheric P_{O_2} at the time of hatching whereas P_{CO_2} was elevated by about 4 torr. Gas tensions in the nests (Figs. 5.3, 5.4) deviated from controls (Figs. 5.5, 5.6) only during the last third of the incubation.

Sampling points away from the nest were rarely different from the controls, even within 25 mm of the nest at the time of maximum embryonic respiration. Occasional high P_{CO_2} (up to 6.0 torr) and low P_{O_2} (as low as 140.5 torr) measured at sampling points away from the nest were assumed to be associated with biological activity in the soil. This indicates that taking "control" samples from points at the depth of the nest need not represent the mean gas tensions in the soil, and may account for the variability in the data. The use of artificial eggs is a more appropriate control.

5.3.2 Water Vapour Conductance of the Shell ($G_{H_2O(s)}$)

Wide standard deviations about the mean rates of weight loss for fresh eggs (Fig. 5.7) indicate large variations in shell conductances between eggs. Fresh eggs lost weight rapidly during the first four hours, after which weight loss stabilized at a lower rate (Fig. 5.8).

Linear regressions, calculated on the percentage weight loss ($g.100g^{-1}$) of all the eggs in each trial for the linear part of each curve and for the mean values (Fig. 5.9) for the same part of the curves, were

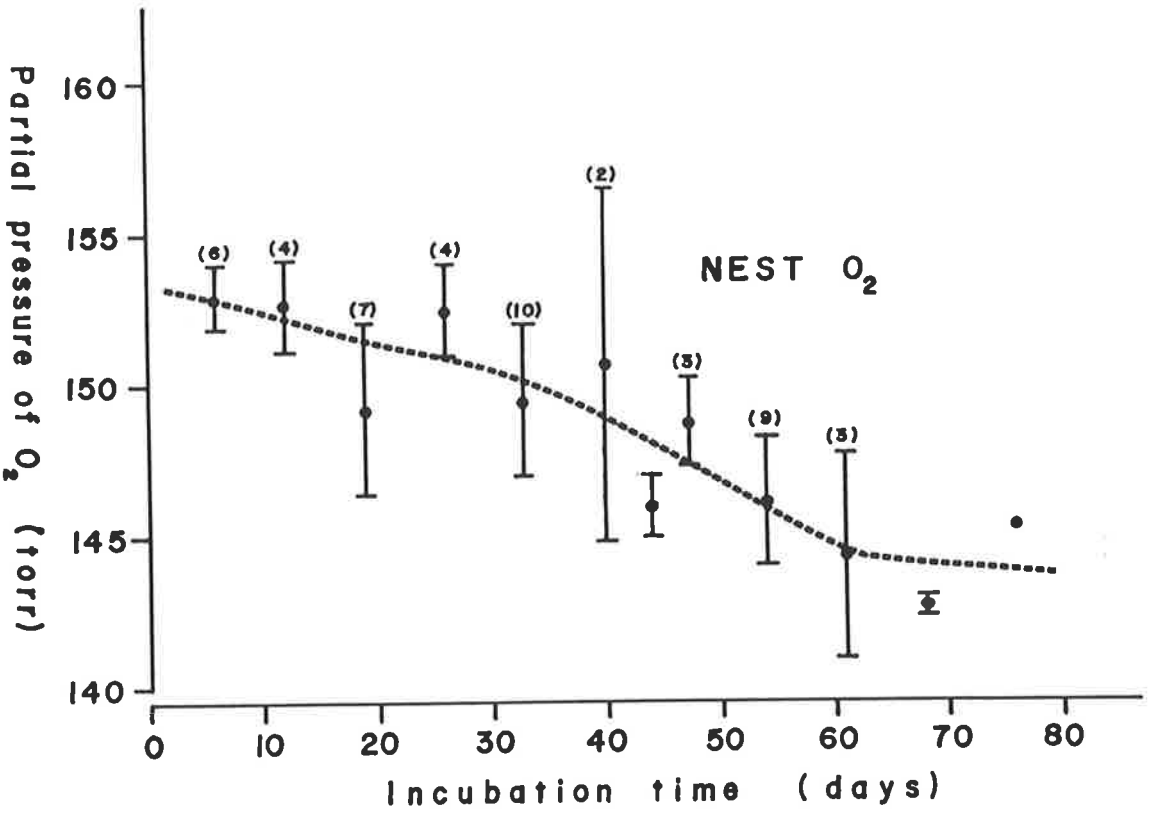


FIGURE 5.3 PARTIAL PRESSURE OF O_2 MEASURED IN THE CENTRE OF THE EGG MASS OF TEN NESTS OF *E. MACQUARII* THROUGHOUT THE INCUBATION PERIOD. BARS REPRESENT ONE STANDARD DEVIATION. LINE FITTED BY EYE. SAMPLE SIZES IN PARENTHESES.

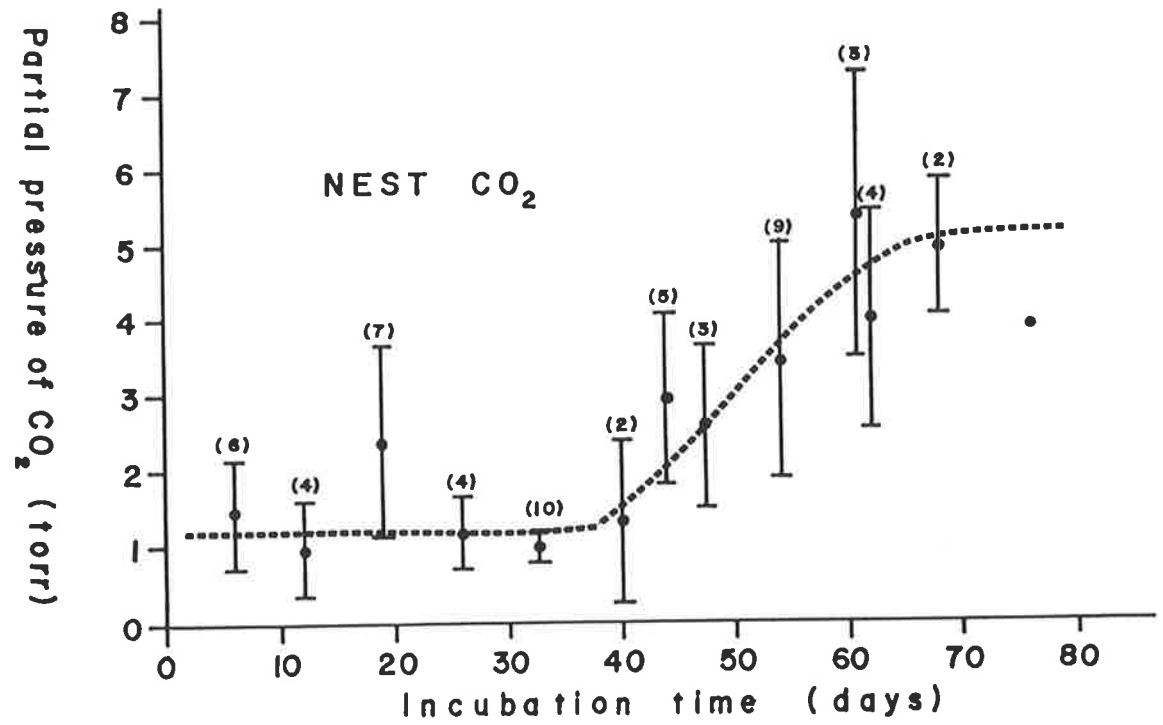


FIGURE 5.4 PARTIAL PRESSURE OF CO_2 MEASURED IN THE SAME NESTS AS IN FIG. 5.3.

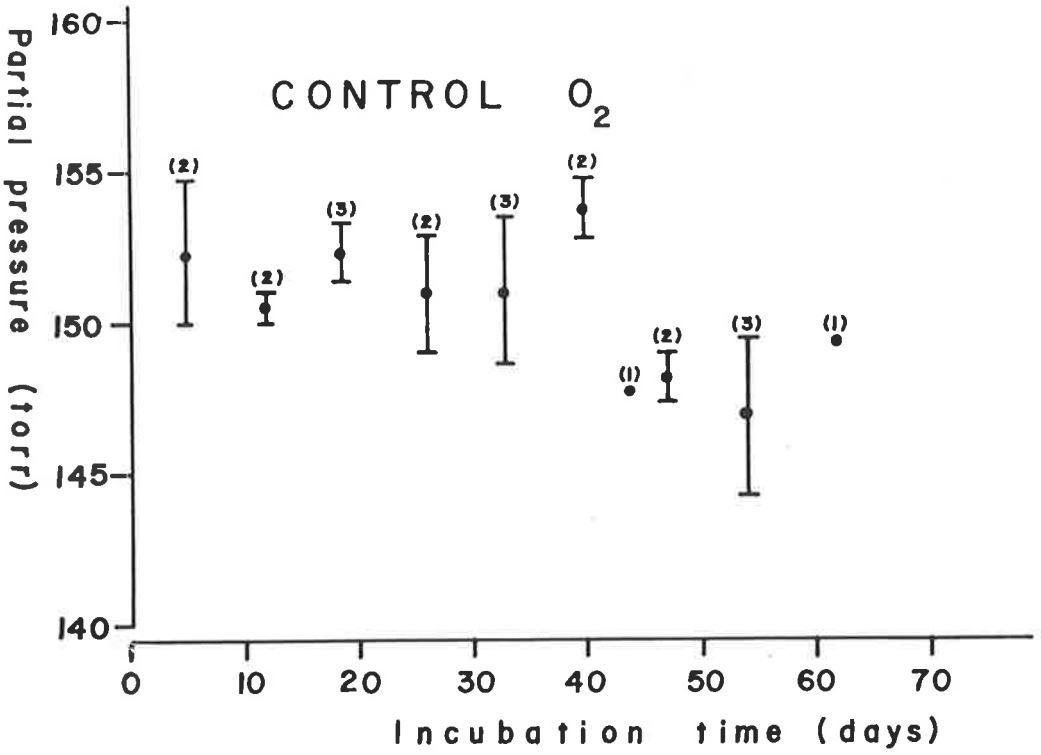


FIGURE 5.5

PARTIAL PRESSURE OF O_2 MEASURED IN THE SOIL AT THE DEPTH OF AN AVERAGE NEST IN TWO PLACES AND IN THE CENTRE OF AN ARTIFICIAL NEST MEASURED AT THE SAME TIME AS THE REAL NESTS IN FIGS. 5.3 AND 5.4. NOTATION AS IN FIG. 5.3.

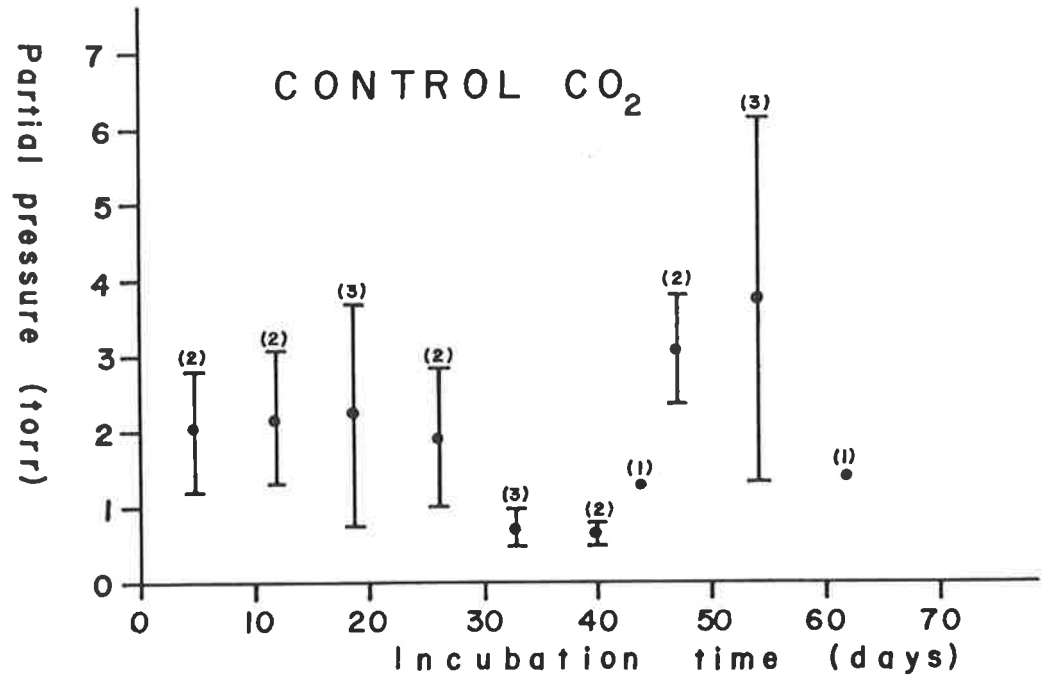


FIGURE 5.6

PARTIAL PRESSURE OF CO_2 AS FOR O_2 IN FIG. 5.5. NOTATION AS IN FIG. 5.3.

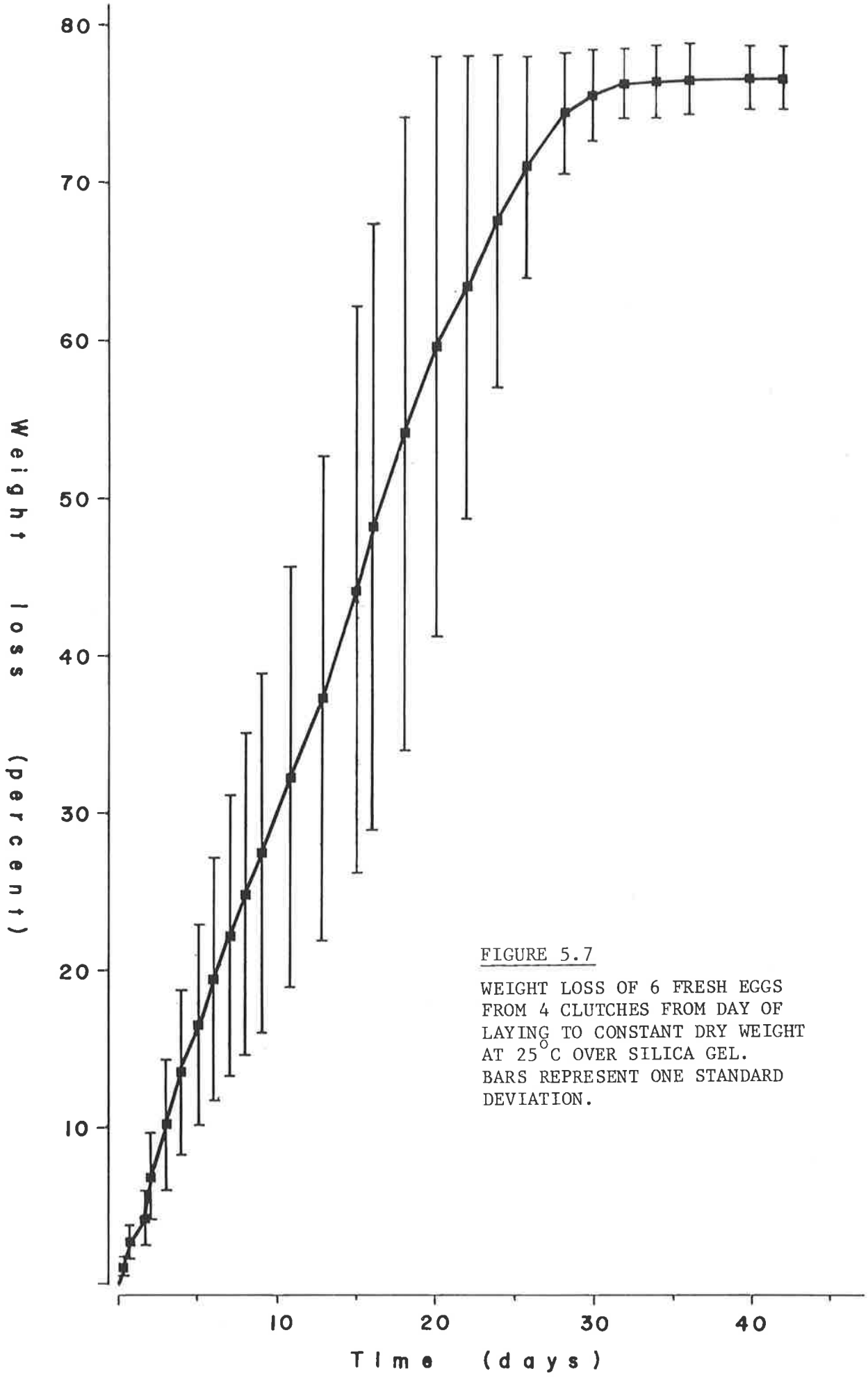


FIGURE 5.7

WEIGHT LOSS OF 6 FRESH EGGS FROM 4 CLUTCHES FROM DAY OF LAYING TO CONSTANT DRY WEIGHT AT 25°C OVER SILICA GEL. BARS REPRESENT ONE STANDARD DEVIATION.

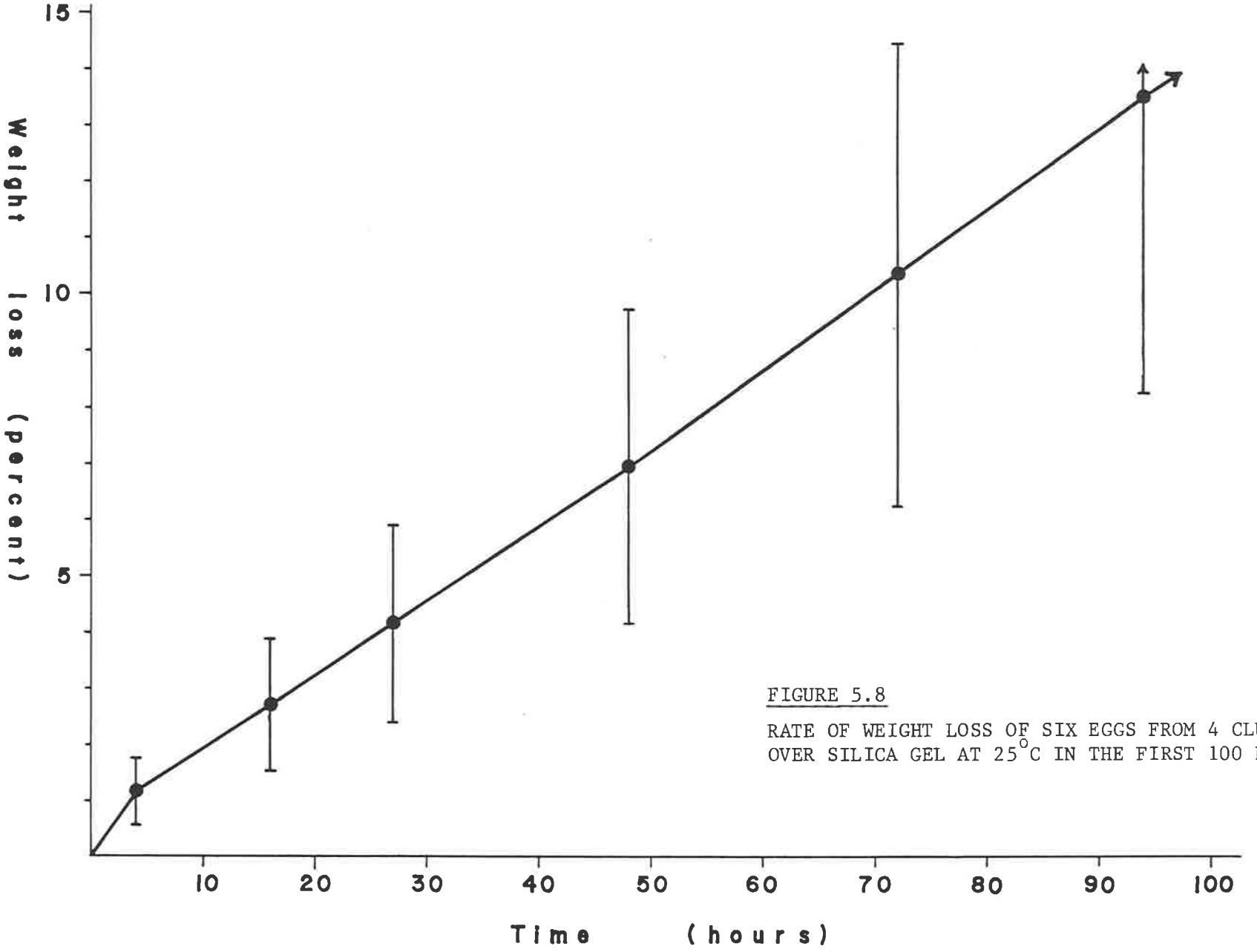


FIGURE 5.8

RATE OF WEIGHT LOSS OF SIX EGGS FROM 4 CLUTCHES OVER SILICA GEL AT 25°C IN THE FIRST 100 HOURS.

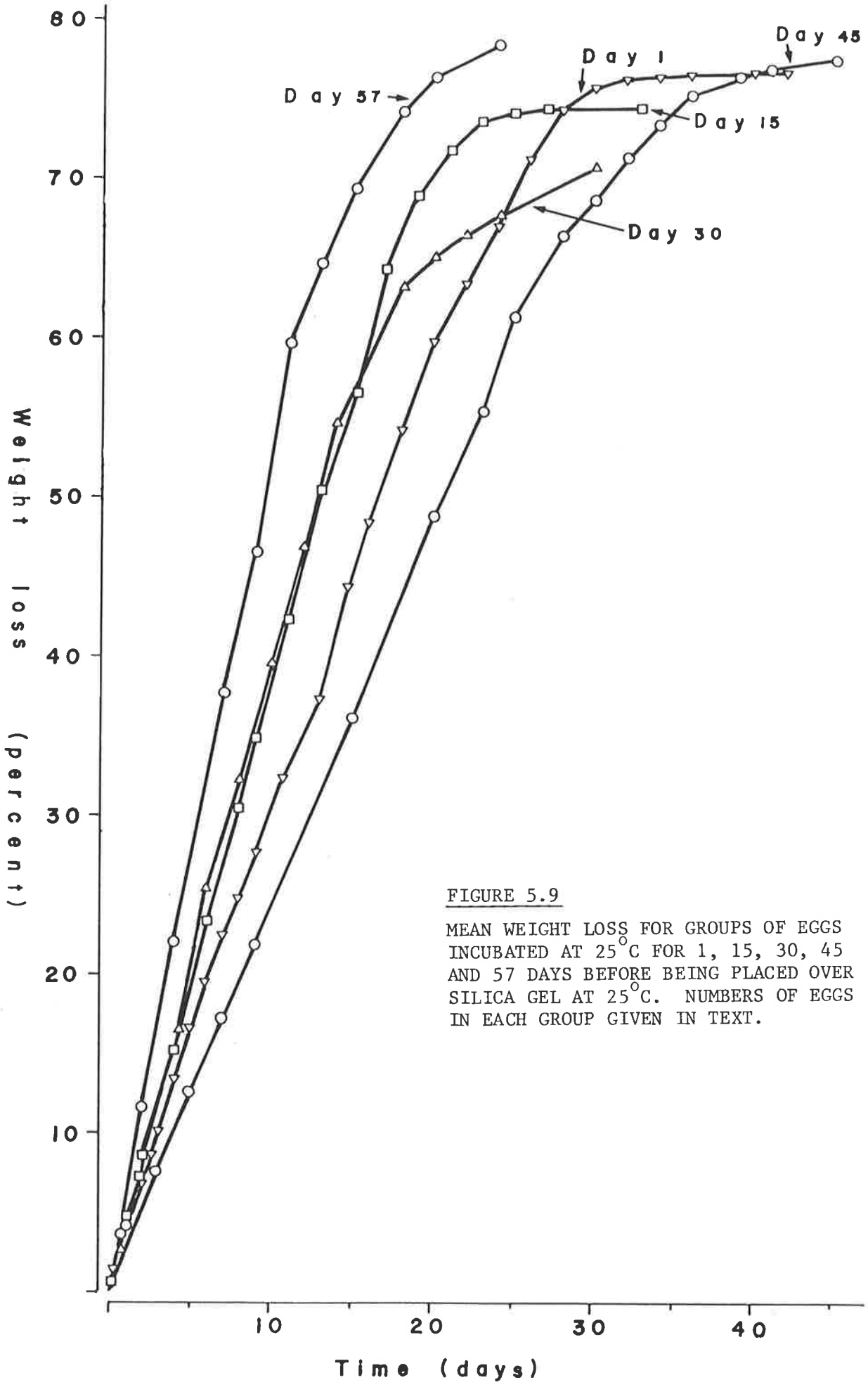


FIGURE 5.9

MEAN WEIGHT LOSS FOR GROUPS OF EGGS INCUBATED AT 25°C FOR 1, 15, 30, 45 AND 57 DAYS BEFORE BEING PLACED OVER SILICA GEL AT 25°C. NUMBERS OF EGGS IN EACH GROUP GIVEN IN TEXT.

statistically equal ($p>0.05$). The regressions were used to calculate the rate of weight loss ($g.100g^{-1}.day^{-1}$) (Table 5.2). Masses of eggs used to measure $G_{H_2O(s)}$ were not significantly different from the mean mass of an *E. macquarii* egg, 10.423 g, ($p>0.20$ in all cases). Therefore 10.423 g was used to calculate the rate of water loss ($mg.\ day^{-1}$) for an average egg of *E. macquarii*. Rates of water loss of all except the day 15 and 30 trials were significantly different from each other (at the 5% level of significance), and increased throughout the incubation in all cases except at 45 days of incubation (Fig. 5.9).

Fertile eggs incubated at 25°C for 57 days lost water at a significantly higher rate ($0.217\ g.100g^{-1}.h^{-1}$) than infertile eggs kept under the same conditions for 57 days ($0.118\ g.100g^{-1}.h^{-1}$) ($t_{(2)52} = 5.929$, $p<0.001$, Fig. 5.10). Infertile eggs lost water at a rate not significantly different from the day 1 eggs ($t = 0.0082$).

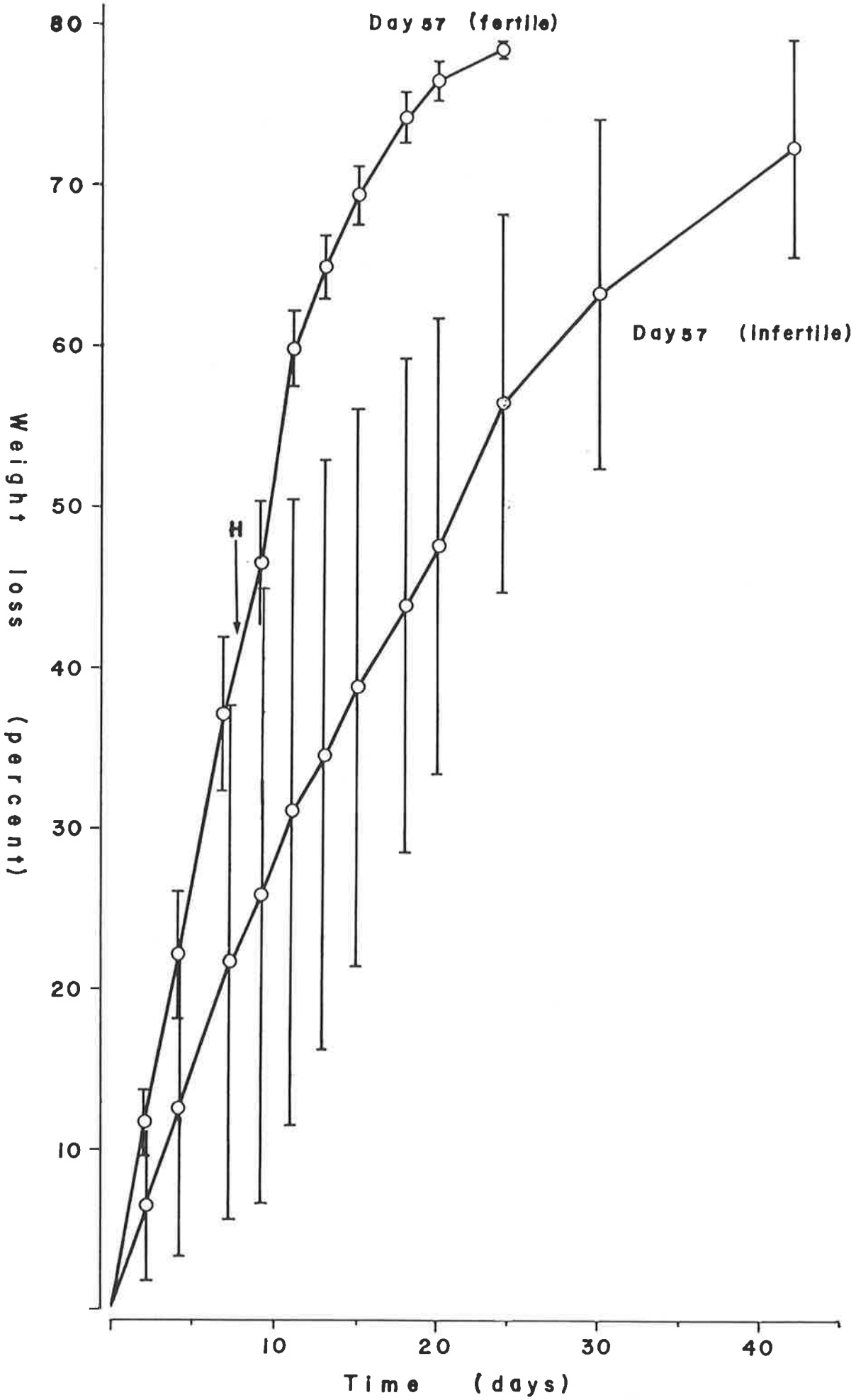
TABLE 5.2

Trial	Slope(a)	y-intercept(b)	n	r^2	Mean Weight Loss		$G_{H_2O(s)}$
					$\%.day^{-1}$	$mg.day^{-1}$	$mg.day^{-1}.torr^{-1}$
Day 1	0.121	1.803	66	0.531	2.9	302.3	12.73
Day 15	0.152	1.743	36	0.750	3.6	375.2	15.79
Day 30	0.158	1.696	28	0.846	3.8	396.1	15.54
Day 45	0.100	0.469	40	0.765	2.4	250.2	10.53
Day 57	0.217	1.160	28	0.963	5.2	542.0	22.82

Linear regressions on straight-line part of curves relating weight loss of eggs over silica gel to time after varying amounts of incubation (1, 15, 30, 45, 57 days). Form of equations is $y = ax + b$ where y = weight loss ($g.100g^{-1}$), x = time (hours), a = slope, b = y-intercept. Mean weight loss was calculated from each equation ($g.100g^{-1}.day^{-1}$) and converted to $mg.day^{-1}$ by multiplying by the mean weight of an egg (= 10.423g). $G_{H_2O(s)}$ was calculated by solving equation 4.1 using mean weight loss ($mg.day^{-1}$) and a P_{H_2O} of 23.756 torr (Weast & Astle, 1981), which assumes that the contents of an egg are saturated with water vapour and the vapour pressure over silica gel is essentially zero (Rahn *et al.*, 1976).

FIGURE 5.10

Weight loss of two groups of eggs incubated at 25 °C for 57 days.
Upper curve represents 6 eggs from 2 clutches that were fertile and developing, lower curve represents 7 eggs from 4 clutches that were infertile or in which the embryo died before any indication of development was apparent from the outside of the egg.
Bars represent one standard deviation.
H indicates the time that one egg hatched.


Day 57 (fertile)
Day 57 (infertile)
H
Weight loss (percent)
Time (days)
80
70
60
50
40
30
20
10
10
20
30
40

Using this empirically determined $G_{H_2O(s)}$ the $G_{O_2(s)}$ and $G_{CO_2(s)}$ were calculated for eggs at the beginning and end of the incubation period (Table 5.3). To provide data comparable with those for avian eggs $G_{H_2O(s)}$ values were corrected for differences in the rate of diffusion of water vapour between 25 and 38°C, and conversion of mg. $day^{-1}.torr^{-1}$ to $cm^3.day^{-1}.torr^{-1}$ using the conversion factors of Paganelli *et al.* (1978).

To calculate the gas tensions experienced by embryonic *E. macquarii* in natural nests at the time of maximum stress, the conductance values were corrected to 33°C (Table 5.3) using the conversion factor of Paganelli *et al.* (1978). This was the maximum temperature recorded in natural nests at the peak of embryonic respiration.

TABLE 5.3 CONDUCTANCE VALUES ($G_{(s)}$) FOR EGGSHELL OF *E. MACQUARII* AT THE BEGINNING AND END OF THE INCUBATION PERIOD CORRECTED TO 33°C.

	Conductance Values $G_{(s)}$ ($cm^3.day^{-1}.torr^{-1}$)		
Trial	G_{H_2O}	G_{O_2}	G_{CO_2}
Day 1	16.06	13.33	10.28
Day 57	28.77	23.88	18.41

The maximum O_2 consumption of a single egg was calculated to be 49.4 $ml.day^{-1}$ at 33°C (from data in Fig. 4.6, and applying a Q_{10} factor of 2.87 (Chapter 4)). Therefore the maximum CO_2 production is 30.1 $ml.day^{-1}$ (using an RE of 0.61 (Chapter 4)). Solving equation 5.1 using $G_{O_2(s)}$ and $G_{CO_2(s)}$ of the eggs prior to hatching (Table 5.3) the maximum ΔP_{O_2} is 2.1 and ΔP_{CO_2} is 1.6 torr.

The surface-specific shell conductance ($K_{(s)}$ $cm^3.sec^{-1}.cm^{-2}.torr^{-1}$)

was calculated using:

$$K_g = \frac{G_g}{A} \qquad (5.2)$$

where A is the surface area (cm^2) of the egg, (Ar *et al.*, 1974). Surface-specific shell conductances were calculated based on a mean egg-shell surface area of 21.547 cm^2 (Chapter 3) (Table 5.4).

TABLE 5.4 SURFACE-SPECIFIC SHELL CONDUCTANCES ($K_{(s)}$) OF EGGS AT THE BEGINNING AND END OF THE INCUBATION PERIOD

	Conductance Values $K_{(s)}$ ($cm^3.sec^{-1}.cm^{-2}.torr^{-1}x10^{-6}$)		
Trial	$K_{H_2O(s)}$	$K_{O_2(s)}$	$K_{CO_2(s)}$
Day 1	8.697	7.219	5.565
Day 57	15.577	12.929	9.970

Conductance is related to total pore area and pore length by

$$G_g = c.\ Dg.\ \frac{Ap}{L} \qquad (5.3)$$

where Ap = total functional pore area (cm^2) and L = length of pore or shell thickness (cm)* (Ar *et al.*, 1974). Dg is the diffusion coefficient of gas, g, in air ($cm^2.sec^{-1}$) and c is a conversion constant. A comparison of equations 5.2 and 5.3 indicates that, for a given shell area, conductance is directly proportional to the functional pore area and inversely proportional to pore length. By combining equations 5.1 and 5.3, and for

---

* In this study and many others on avian eggs, shell thickness and pore length have been used interchangeably (e.g. Tullett & Board, 1976). It was pointed out that this is not strictly correct because of the mammillary cones in avian eggs (Hanka *et al.*, 1979; G. Packard *et al.*, 1979b) (= conical tips in chelonian eggs) and can lead to errors in calculations that require a knowledge of pore length.

the conditions used in this experiment (i.e. T = 298° K, ΔP_{H_2O} = 23.756 torr),

$$Ap = \frac{G_{H_2O(s)} \cdot L}{23.42} \qquad (5.4)$$ (Ar *et al.*, 1974).

G_{H_2O} and L for fresh eggs at 298° K were 12.73 mg.day^{-1}.torr^{-1} and 0.0173 cm. The units of the denominator are mg.day^{-1}.cm^{-1}.torr^{-1}. Therefore the total functional pore area = 0.940 mm^2, or 0.0436% of the total shell area. Assuming the mean pore diameter to be 18.5 μm (Chapter 3), then solving equation 5.4, an average egg (i.e. 10.423 g) has 3,500 pores.

5.3.3 O_2 and CO_2 Conductance of Shell and Membranes ($G_{O_2(ms)}$, $G_{CO_2(ms)}$)

The rate of diffusion of O_2 and CO_2 through isolated eggshell and shell membranes was calculated using the equation

$$\dot{V}_g = \dot{V}_{N_2} \cdot F_g \qquad (5.5)$$

where $\dot{V}_g$ is the rate of diffusion of the gas (O_2 or CO_2) (cm^3.min^{-1}), $\dot{V}_{N_2}$ is the rate of flow of N_2 through the egg (cm^3.min^{-1}) and F_g is the fraction of either O_2 or CO_2 measured in the sample after passing through the egg (dimensionless). Because the egg was vented to the atmosphere, the partial pressure difference of O_2 or CO_2 across the shell was assumed to be equal to the atmospheric pressure less the water vapour pressure at 30°C (31.824 torr, Weast and Astle, 1981).

$G_{O_2(ms)}$ was zero when the eggs were first laid. Conductance increased from day 2 to day 12. Then there was a wide scatter in the data with all $G_{O_2(ms)}$ values greater than 1.1 cm^3.day^{-1}.torr^{-1} until day 35 when conductance fell slightly (Fig. 5.11). Non-developing eggs had no measurable conductance when measured 8, 60 and 61 days after laying. There was a measurable $G_{CO_2(ms)}$ on day 1. It increased until about day

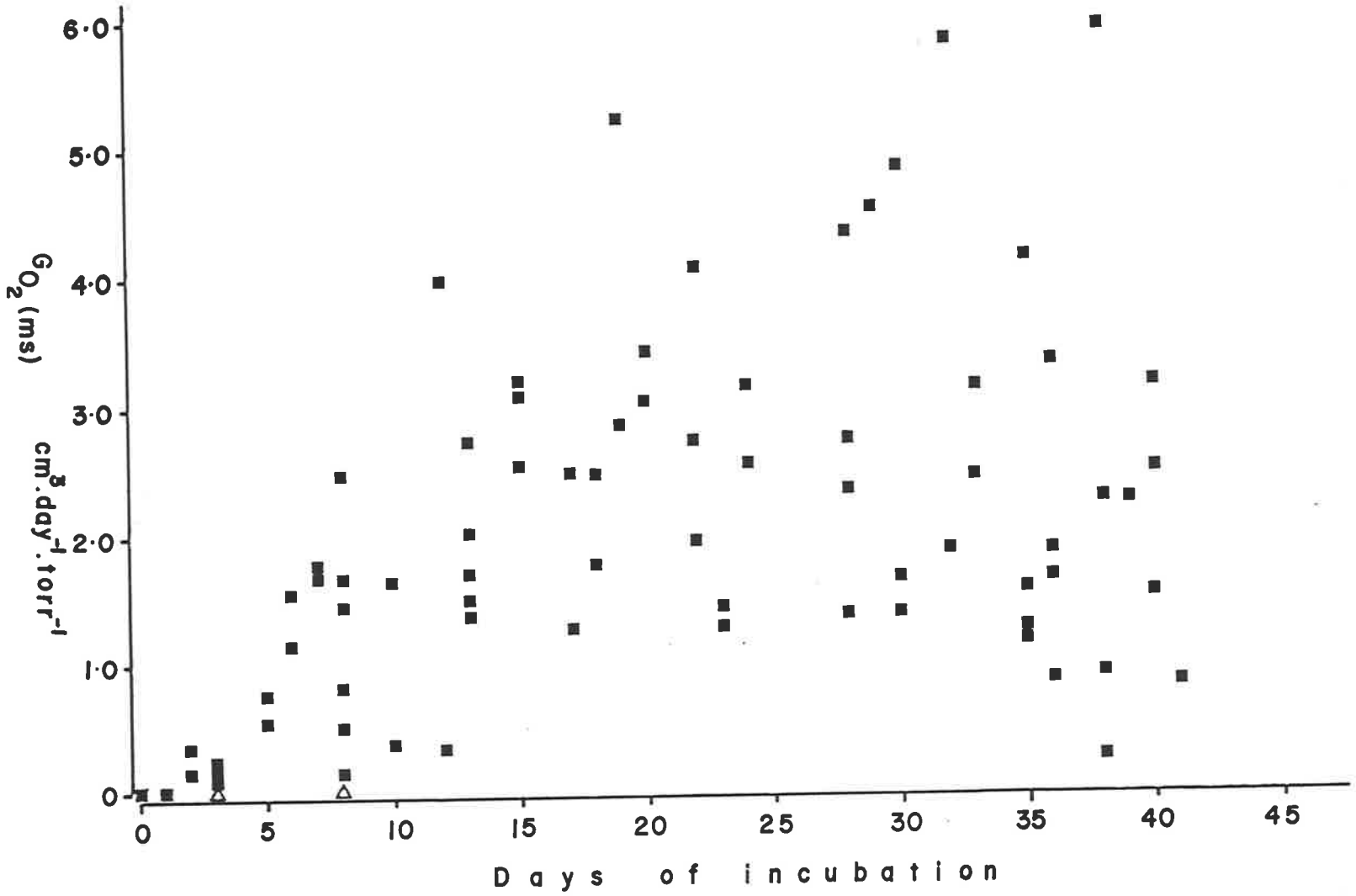


FIGURE 5.11 OXYGEN CONDUCTANCE OF EGGSHELL AND MEMBRANES ($cm^3.day^{-1}.torr^{-1}$) OF 81 EGGS FROM FOUR CLUTCHES THROUGHOUT THE INCUBATION PERIOD. Δ = NON-DEVELOPING EGGS.

25 and then decreased slightly until hatching (Fig. 5.12).

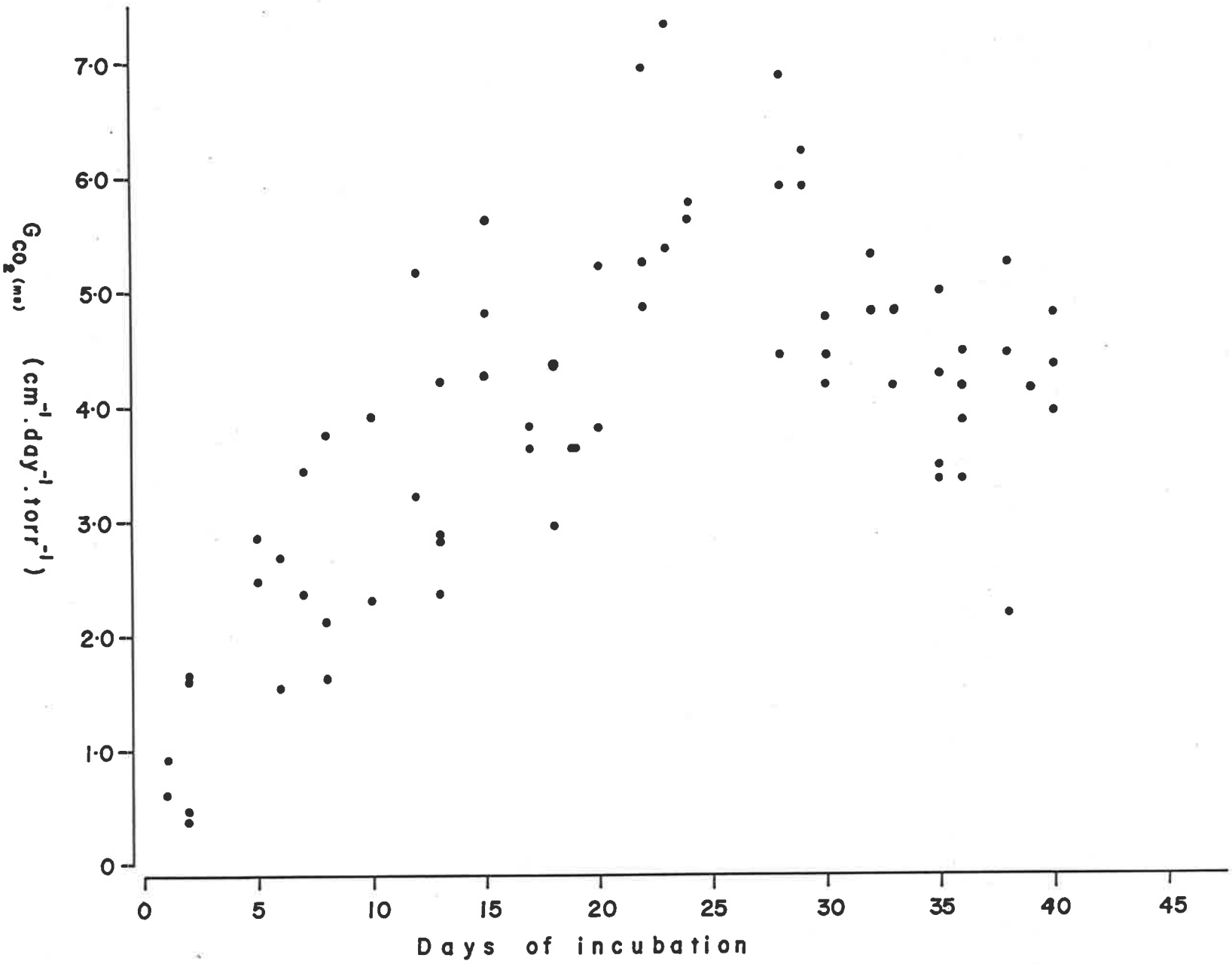
When laid, the eggs are translucent, the yolk giving the egg a yellowish tinge. The white patch forms on the upward facing surface of developing eggs between 12 and 30 hours after laying. It increases rapidly until it forms a saddle-shaped patch on the top of the shell (Fig. 5.13). The two sides of the saddle usually meet around the equator of the egg on day 3 or 4 to form an opaque band as in *Alligator mississippiensis* (Ferguson, 1982). In some eggs the whole shell eventually becomes opaque and white; in others one or both ends remain translucent until hatching. The first egg became completely opaque after 12 days.

Because of variations in the opaque white patch between eggs, the conductance data were plotted against the area of the white patch, expressed as a percentage of total shell area (Figs. 5.14, 5.15). There is a positive correlation between conductance and the area of the white patch with conductance increasing only slowly until after the white patch covers 30% of the shell. Then it increases more rapidly until the white patch covers 100% of the shell. The mean value of $G_{O_2(ms)}$ for eggs with the white patch covering 100% of the shell is 2.642 ± 1.088 $cm^3.day^{-1}.torr^{-1}$ (n = 16) and $G_{CO_2(ms)}$ is 4.742 ± 1.328 $cm^3.day^{-1}.torr^{-1}$ (n = 15) at 30°C; corrected to 33°C these measurements are $G_{O_2(ms)}$ = 2.655 and $G_{CO_2(ms)}$ = 4.765 $cm^3.day^{-1}.torr^{-1}$.

Maximum P_{O_2} across the shell and membranes was calculated as 19.0 torr and for P_{CO_2} as 7.5 torr.

The mean conductance values, corrected to 33°C, for the four eggs dried over silica gel ($G_{O_2(ms)}$ = 5.554 and $G_{CO_2(ms)}$ = 6.692 $cm^3.day^{-1}$.

FIGURE 5.12 Carbon dioxide conductance of eggshell and membranes ($cm^3.day^{-1}.torr^{-1}$) of 68 eggs from three clutches throughout the incubation period.


7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
$G_{CO_2(ms)}$ ($cm^{-1}.day^{-1}.torr^{-1}$)
0
5
10
15
20
25
30
35
40
45
Days of incubation

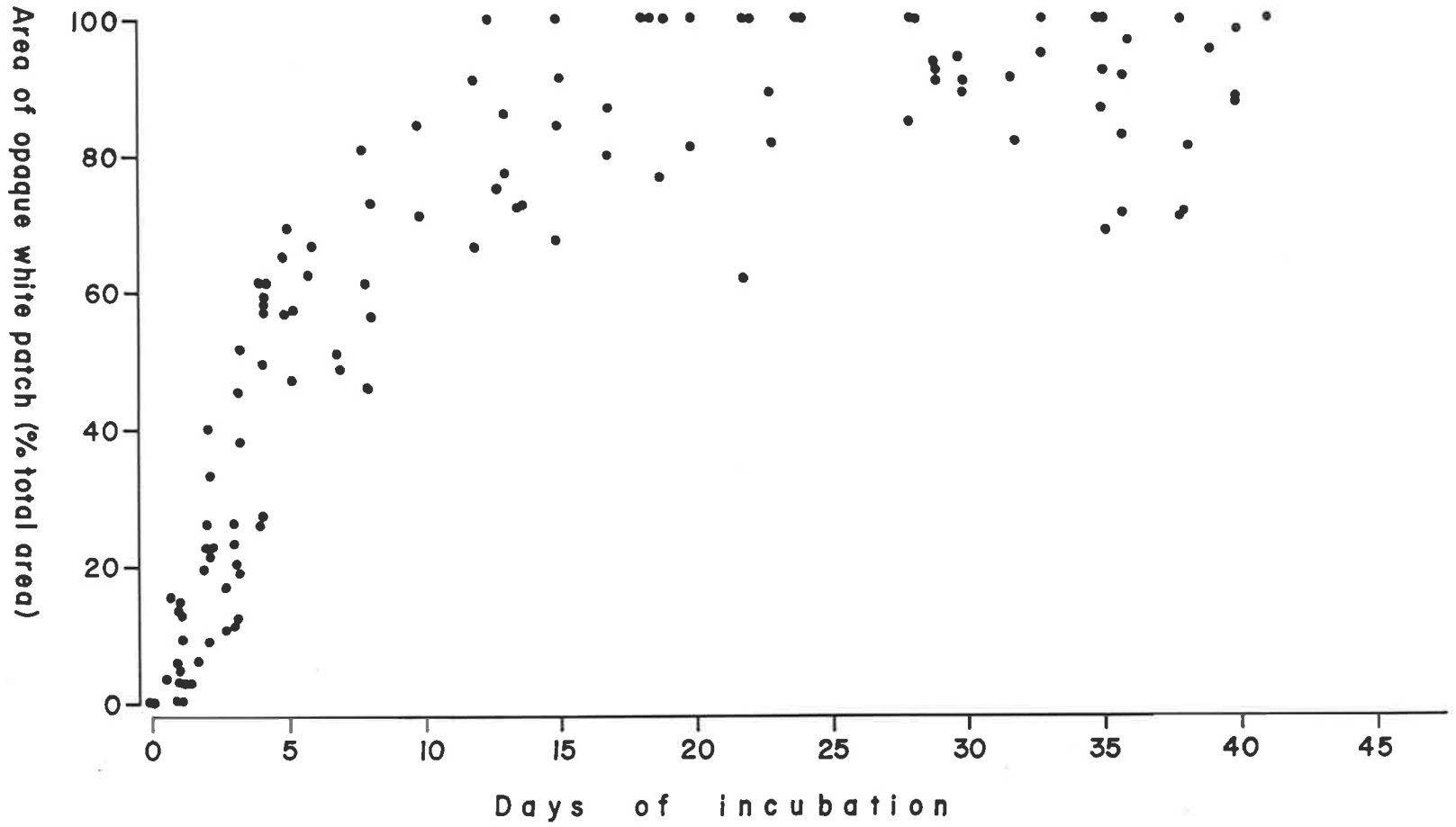


FIGURE 5.13 ONE HUNDRED AND FIFTEEN MEASUREMENTS OF THE AREA OF THE WHITE PATCH AS A PERCENTAGE OF THE TOTAL SHELL AREA OF 84 EGGS THROUGHOUT THE INCUBATION PERIOD AT 30°C.

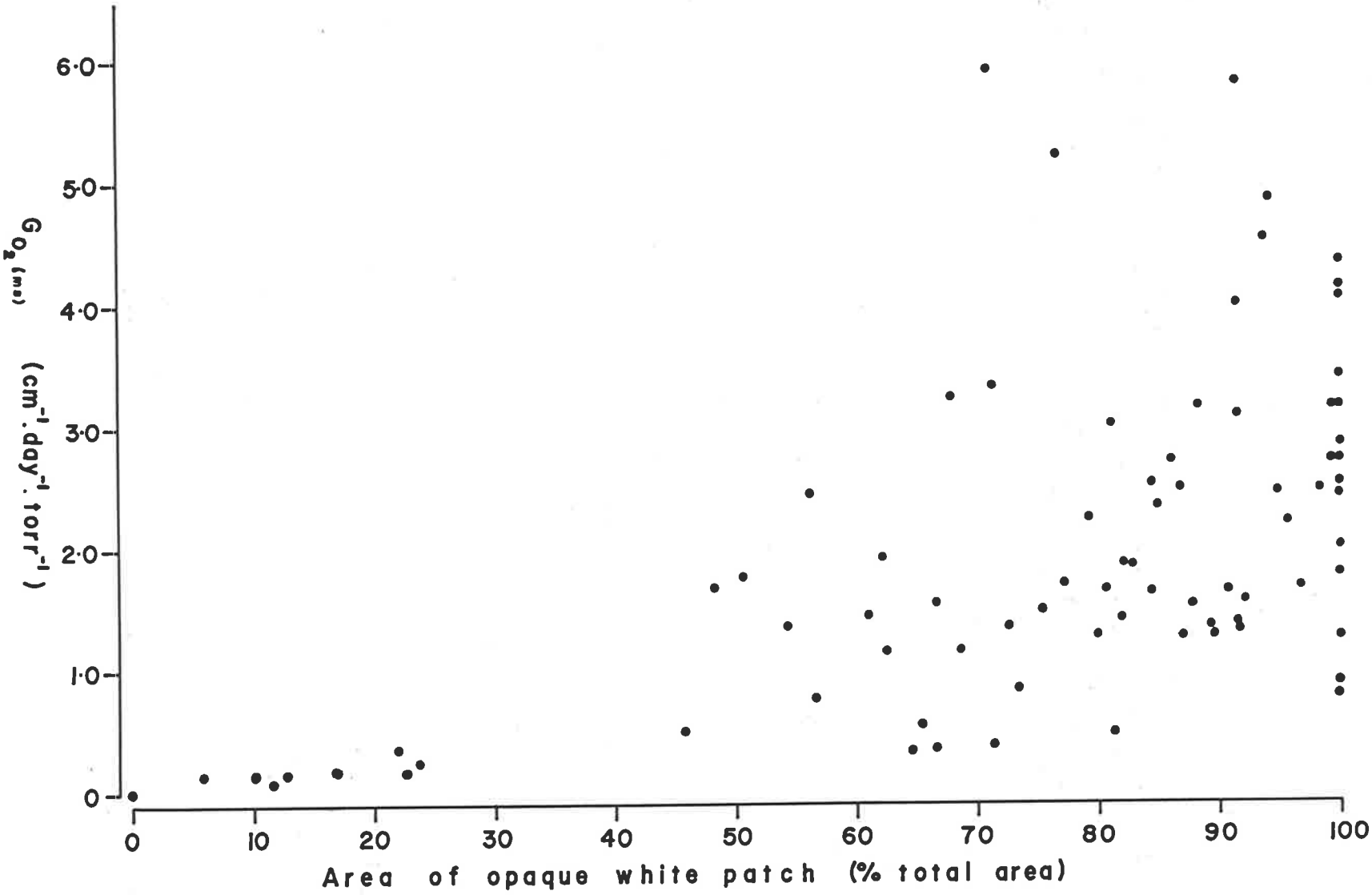
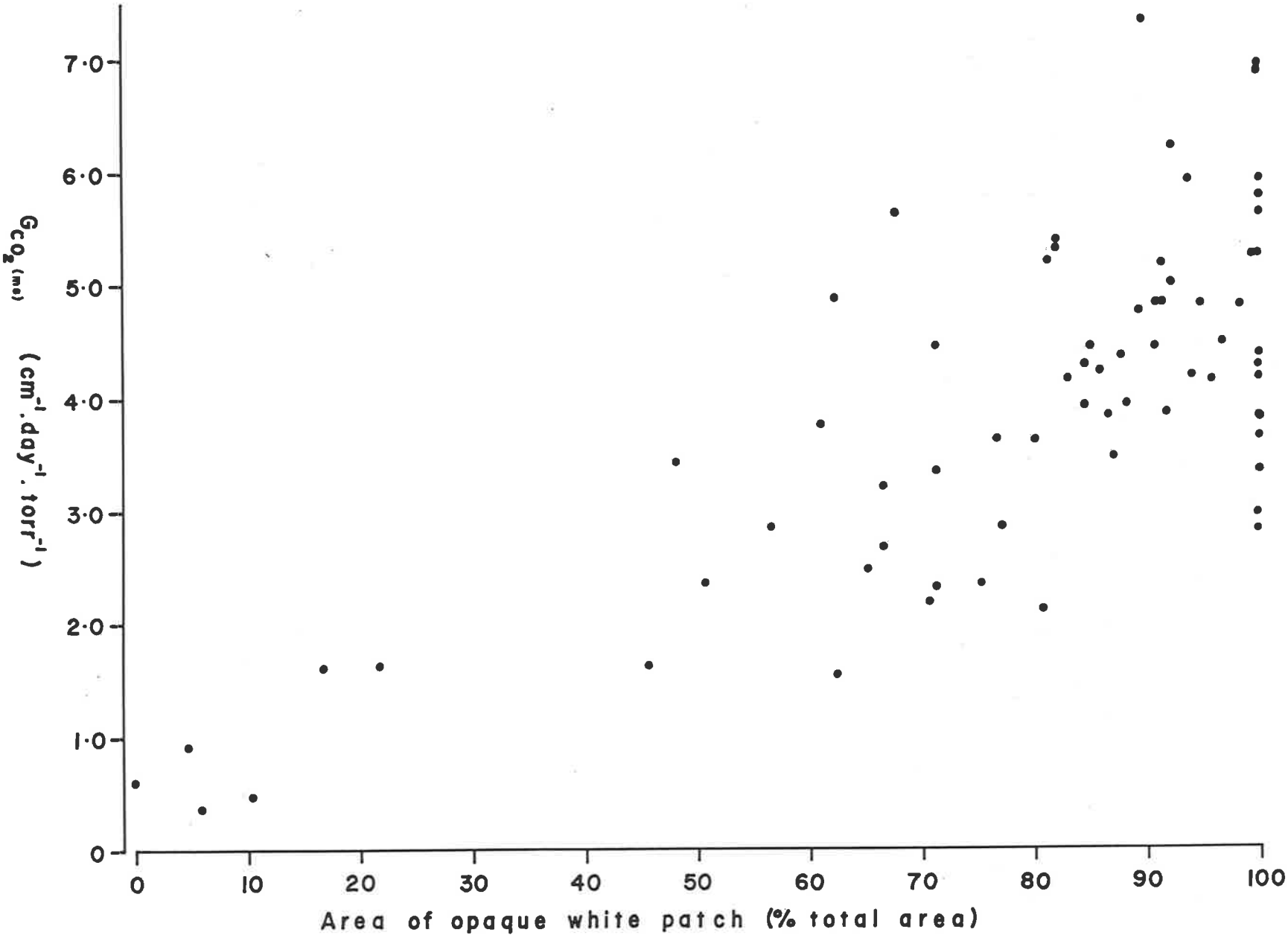


FIGURE 5.14 G_{O_2} OF THE EGGSHELL AND MEMBRANES PLOTTED AGAINST THE AREA OF THE WHITE PATCH FOR 86 SEPARATE EGGS.

FIGURE 5.15 G_{CO_2} of the eggshell and membranes plotted against the area of the white patch for 68 separate eggs.


7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
$G_{CO_2(m)}$ ($cm^{-1}.day^{-1}.torr^{-1}$)
0
10
20
30
40
50
60
70
80
90
100
Area of opaque white patch (% total area)

$torr^{-1}$) gave partial pressure differences across the eggshell of 9.1 and 5.4 torr.

The surface-specific shell conductances ($K_{O_2(ms)}$ and $K_{CO_2(ms)}$) were calculated for each egg individually and, because the eggs were similar in size, the pattern of change of $K_{O_2(ms)}$ and $K_{CO_2(ms)}$ throughout the incubation period was similar to that of $G_{O_2(ms)}$ and $G_{CO_2(ms)}$. The mean $K_{O_2(ms)}$ was 1.359 (± 0.568) x 10^{-6} $cm^3.sec^{-1}.cm^{-2}.torr^{-1}$ (n = 16) and the mean $K_{CO_2(ms)}$ was 2.411 (± 0.618) x 10^{-6} $cm^3.sec^{-1}.cm^{-2}.torr^{-1}$ (n = 4).

The water content of translucent shell was significantly greater than that of opaque shell ($t_{(2),9}$ = 4.721, $p<0.002$) (Table 5.5). In all cases the water content was less in the opaque shell than in translucent shell from the same egg, but there was considerable variation between eggs.

TABLE 5.5 WATER CONTENT OF OPAQUE AND TRANSLUCENT SHELL, AS A PERCENTAGE OF TOTAL WET WEIGHT (n = 6).

Shell	$\bar{x}$	± S.D.	Range
Opaque	15.0	2.06	12.4 - 18.4
Translucent	20.4	2.28	17.2 - 22.5

5.3.4 Gas Tensions Experienced by the Embryo

The gradient in gas tensions across the shell membranes was calculated using

$$\Delta P_{g(m)} = P_{g(ms)} - \Delta P_{g(s)} \qquad (5.6)$$

where $\Delta P_{g(m)}$ is the difference in the tension of gas, g, across the shell membranes, $\Delta P_{g(ms)}$ is across the shell and membranes and $\Delta P_{g(s)}$ is across the shell. The maximum ΔP_{O_2} and ΔP_{CO_2} for each of the resistances to dif-

fusion between a developing embryo and the atmosphere were combined (Fig. 5.16) to calculate the P_{O_2} and P_{CO_2} experienced by the embryo (122 torr and 15 torr). This calculation was based on the assumption that all water vapour was being lost from the outer shell membrane through gas filled pores in the calcareous shell. If the opaque white patch on the eggs of *E. macquarii* forms as the pores in the shell dry out then this assumption is met only when it covers 100% of the surface of the shell which occurred in many eggs by the time of maximum O_2 consumption (Fig. 5.13). The assumption must be met in order to validly calculate G_{O_2} and G_{CO_2} from water loss data (Wangensteen and Rahn, 1970/71; Paganelli *et al.*, 1971). It also implies that the outer shell membrane remains wet. If it is dry then the calculations will give the conductance of the shell and outer shell membrane combined. For clarity, I have assumed that the outer and inner shell membranes form one discrete diffusion barrier and the shell another. This implies that the outer shell membrane is wet; if it is dry, however, as in chicken eggs, its contribution as a barrier to diffusion is negligible (Wangensteen *et al.*, 1970/71; Paganelli, 1980).

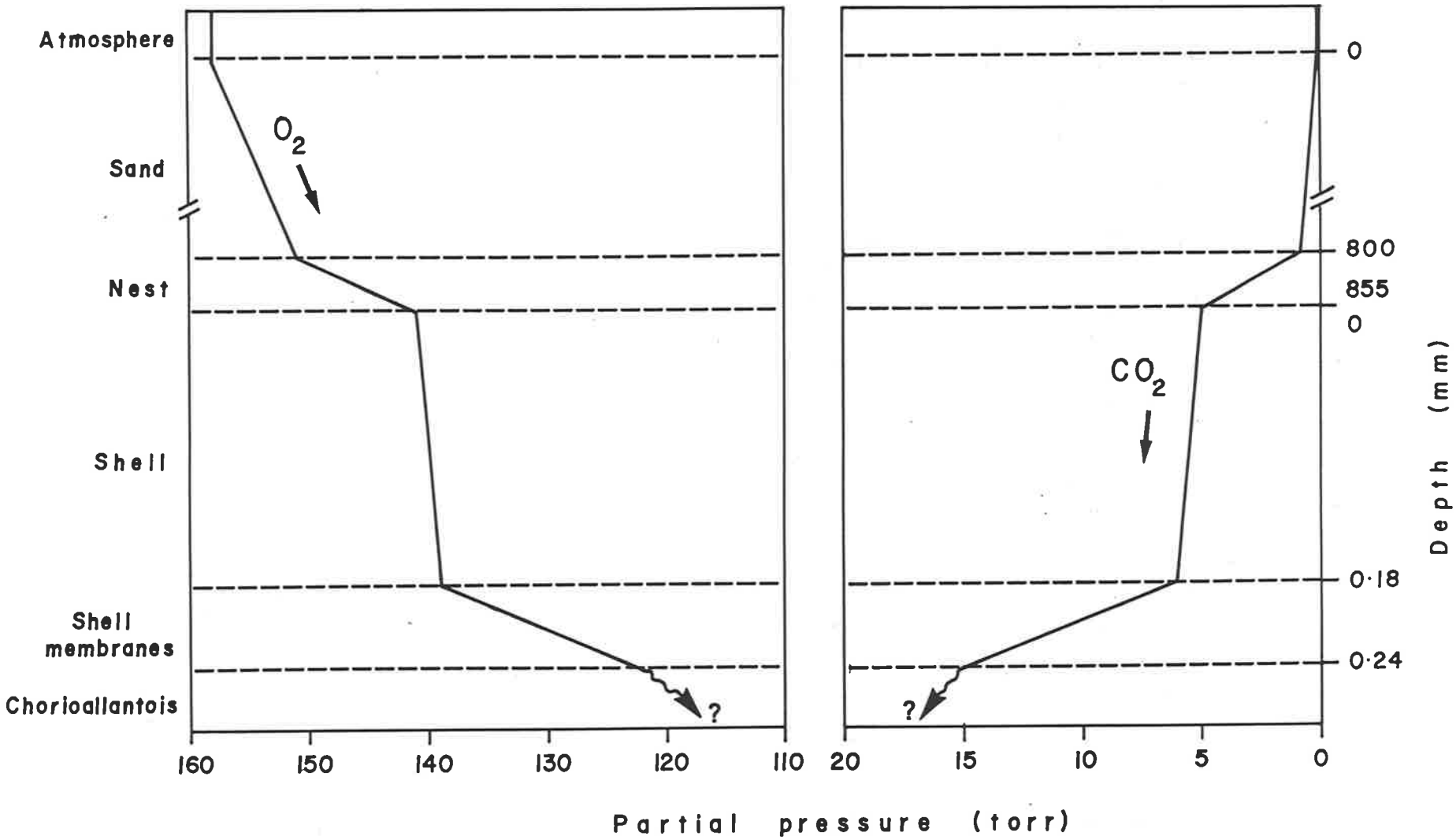


FIGURE 5.16 GAS TENSIONS MEASURED OR CALCULATED BETWEEN THE ATMOSPHERIC AIR AND THE DEVELOPING EMBRYO OF AN EGG OF *E. MACQUARII* MORE THAN 80% OF THE WAY THROUGH THE INCUBATION PERIOD.

5.4 DISCUSSION

5.4.1 Gas Tensions Experienced by Naturally Developing Embryos

Gas tensions in nests of *E. macquarii* are closer to atmospheric levels than in marine turtle nests. However, lower shell conductances in *E. macquarii* compensate for this and gas tensions at the chorioallantois in embryonic *E. macquarii* (P_{O_2} ≈ 120 torr, P_{CO_2} ≈ 15 torr, Fig. 5.16), are similar to those in marine turtles (P_{O_2} ≈ 80 - 100 torr, P_{CO_2} ≈ 40 - 60 torr in *Chelonia mydas*; P_{O_2} ≈ 110 - 120 torr, P_{CO_2} ≈ 20 - 40 torr in *Caretta caretta*).

The original comparison by Ackerman (1977) was between gas tensions in marine turtle nests and the airspaces of avian eggs which were thought to reach similar values just prior to hatching (P_{O_2} ≈ 100 torr, P_{CO_2} ≈ 40 torr; Rahn *et al.*, 1974, 1979). However, D. Vleck *et al.* (1980) calculated the gas concentrations in the air space of avian eggs do not all reach the same endpoint, but are related to the fresh egg mass (P_{CO_2}, for example, ranges from 20 torr in the ostrich to 68 torr in the zebra finch). Large eggs have thicker membranes than small eggs and the added diffusion barrier may compensate for relatively high conductances of the eggshell and outer shell membrane (D. Vleck *et al.*, 1980). The relationship between egg weight and gas tensions obtained by D. Vleck *et al.* (1980) may have been an artefact of the technique used to determine air cell gas tensions (Tullett & Deeming, 1982), and further analysis is needed. Ninety-six percent of the total ΔP_{O_2} (52 torr) and 38% of ΔP_{CO_2} (2.6 torr) across the inner shell membrane, chorioallantoic membrane and endothelium in chicken eggs is due to blood shunting in the vessels of the chorioallantoic membrane (Piiper *et al.*, 1980). The shunt, together with changes in embryonic heart rate (Laughlin, 1978) may act to maintain relatively stable blood gas tensions (Piiper *et al.*, 1980). Consequently, changes in aircell gas tensions of the order found by D. Vleck *et al.*

(1980) probably have little effect on the embryo.

Ackerman (1977) assumed that elevated P_{CO_2} and depressed P_{O_2} in the nests of marine turtles provided gas tensions just prior to hatching similar to those normally experienced in the lung, as in avian eggs (Rahn *et al.*, 1979). Measurements of gas tensions in the lungs of adult *Pseudemys scripta* and *Testudo graeca* showed P_{O_2} to be about 120 torr and P_{CO_2} about 20 torr during normal breathing (Burggren & Shelton, 1979), close to the gas tensions beneath the inner shell membrane of *E. macquarii* just prior to hatching (Fig. 5.16). This supports the assumption of Ackerman (1977).

Applying equation 5.1 to data from Lutz *et al.*, (1980) for eggshell of *Crocodylus acutus* I calculated ΔP_{O_2} of 16.5 torr and ΔP_{CO_2} of 15.1 torr at 33°C, the incubation temperature of the eggs (Dunbar-Cooper & Lutz, 1981), across eggshell that had lost 30% of its water. I assumed that conductance measurements of the eggshell and membranes from infertile eggs of *C. acutus* that had lost 30% of their water approximated the ΔP_{O_2} and ΔP_{CO_2} across the shell and membranes of developed eggs because the opaque shell of *E. macquarii* contains 26% less water than translucent shell (Table 5.5). P_{CO_2} and P_{O_2} in the nests of *C. acutus* reach 20 torr and 116 torr respectively (Dunbar-Cooper & Lutz, 1981). Hence the subshell CO_2 and O_2 tensions should be about 35 torr and 100 torr respectively. This is compatible with the prediction of Ackerman (1977).

The P_{O_2} and P_{CO_2} tensions in the nests of *C. acutus* (Dunbar-Cooper & Lutz, 1981) are intermediate between those of *E. macquarii* and marine turtles (Ackerman, 1977). This species has a clutch weight and maximum O_2 consumption similar to that of marine turtles (Seymour, 1979), but the nest gas tensions are between those of marine turtle nests and the atmo-

sphere. Accordingly, the partial pressure change across the shell is higher (i.e. conductance is lower) in *C. acutus* (Harrison *et al.*, 1978) than in marine turtles (Prange & Ackerman, 1974). This indicates that selective pressures have matched shell conductances to nest gas tensions to prevent intolerably low O_2 tensions and high CO_2 tensions from developing in the eggs. Similar selective pressures have modified the eggshell conductances of mallee fowl and brush turkeys which also incubate their eggs underground (Seymour & Ackerman, 1980).

TABLE 5.6

	E. macquarii	*C. acutus*	Marine Turtles
P_{O_2} (nest)	140	115[a]	80 - 100[c]
ΔP_{O_2} (shell)	20	15[b]	2[d]
P_{O_2} (subshell)	120	100	80 - 100
P_{CO_2} (nest)	5	20[a]	40 - 60[c]
ΔP_{CO_2} (shell)	10	15[b]	2[d]
P_{CO_2} (subshell)	15	35	40 - 60

Approximate nest gas tensions (torr) ($P_{O_2\ (nest)}$ and $P_{CO_2\ (nest)}$), gradient in gas tensions across the eggshell (torr) ($\Delta P_{O_2\ (shell)}$ & $\Delta P_{CO_2\ (shell)}$) and subshell gas tensions (torr) experienced by the embryo.
References: a. Dunbar-Cooper & Lutz (1981); b. see text; c. Ackerman (1977); d. Prange & Ackerman (1974).

5.4.2 Conductance of the Shell and Membranes

It is obvious that the shell membranes offer a higher resistance to the diffusion of O_2 and CO_2 in the eggs of *E. macquarii* than does the shell itself. The G_{O_2} of the eggshell in *E. macquarii* is about ten times higher than that of the eggshell plus shell membranes, whereas the G_{CO_2}

is only about four times as high, just prior to hatching. In eggs of the marine turtle, *Caretta caretta*, and the American crocodile, *C. acutus*, the rates of water loss were also about ten times that predicted from the G_{O_2} of the shell (Ackerman, 1980; Lutz *et al.*, 1980). Because the shell membranes are such a barrier to the diffusion of gases in reptiles, the G_{H_2O} measured in eggs under constant conditions of humidity and temperature cannot be used to calculate the gas tensions adjacent to the embryonic tissue (cf. avian eggs: e.g. D. Vleck *et al.*, 1980).

G_{H_2O} measured via weight loss in the eggs of the American alligator, *Alligator mississippiensis*, and the soft-shelled turtle, *Trionyx spiniferus*, were 4.2 and 5.5 times as high as predicted for avian eggs of the same size (G. Packard *et al.*, 1979b) and twice the predicted value in *C. acutus* (Harrison *et al.*, 1978; Lutz *et al.*, 1980), using the equations of Ar *et al.* (1974). The snapping turtle, *Chelydra serpentina*, which lays soft-shelled eggs, had a G_{H_2O} 54.6 times the predicted value for an avian egg of the same size. The hard-shelled eggs of some geckoes have lower conductances than any other reptilian or avian eggs (Dunson & Bramham, 1981), including *Testudo graeca*, which I calculated to have a shell conductance one third that of an avian egg of similar size from the data of Young (1950). The low conductance, and the presence of a cuticle on the surface of the eggshell of *T. graeca* (Young, 1950) indicate that these eggs may develop in drier conditions than those of other chelonians.

Combined, these results indicate that the shell's resistance to water vapour diffusion varies amongst reptiles. In species with soft-shelled eggs (e.g. *C. serpentina*), it contributes far less resistance than in species with hard-shelled eggs.

The ratio of G_{CO_2} to G_{O_2} was 1.8 in eggs of *E. macquarii* with white

patch covering the whole shell. Because CO_2 diffuses through water 20 - 25 times faster than O_2 (Dejours, 1975) but only 0.78 times as fast through air (Wangensteen & Rahn, 1970/71) 93.4 - 94.5% of the conductance occurs through gas-filled pores in these eggs, the rest passing in aqueous solution. The conductance ratio in newly laid eggs could not be calculated because the G_{O_2} was so low, indicating that more, if not all, of the conductance occurs in the aqueous phase until the white patch forms.

There was considerable variation in the conductance measurements on the eggshell and shell plus membranes in the eggs of *E. macquarii*. However, the amount of variation is not greater than in the eggs of chickens (Romijn & Roos, 1938; Wangensteen *et al.*, 1970/71; Kutchai & Steen, 1971; Paganelli *et al.*, 1971; Tullett & Deeming, 1982), marine turtles (Ackerman & Prange, 1972) or alligators (G. Packard *et al.*, 1979b). Thus large variations in eggshell conductance are typical. Contrary to Rahn *et al* (1976) avian eggs, and probably reptilian eggs, have a considerable "safety margin" in the amount of water they are able to lose without adversely affecting the hatching rate (Simkiss, 1980b).

5.4.3 The Opaque White Patch

The development of the opaque white patch was a striking characteristic of the developing eggs of *E. macquarii*. The shell and membranes of the white patch contain less water than the adjacent translucent shell (Table 5.5). Although the chorioallantoic membrane of the developing embryo lies directly beneath the white patch, it extends beyond the extra-embryonic membranes, especially early in incubation. White patches do not form on infertile eggs. It is therefore concluded that the white patch is a regional drying of the shell, influenced by the developing embryo and related to the respiratory requirements of that embryo. No other factors,

such as the erosion craters in the eggs of alligators (Ferguson, 1982), are implicated in this case. The shell membranes of infertile chicken eggs lose very little water whereas those in fertile eggs lose over 40% which suggests that the embryo influences the drying of the membranes (Kutchai & Steen, 1971). No white patch and no detectable G_{O_2} develop in infertile eggs of *E. macquarii* at any time during the incubation period, suggesting that the same may also be true for chelonian eggs.

Although the white patch influences the conductance of the eggshell and membranes (Figs. 5.14, 5.15) and covers the whole shell by day 12 (Fig. 5.13), maximum O_2 demand does not occur until day 37 at 30°C (Fig. 4.3). Hence the increase in conductance with growth of the white patch precedes that necessary to supply the O_2 requirements of the embryo as it develops. Increased conductance of the eggshell and membranes of fertile chicken eggs after two days also exceeds the respiratory requirements of the developing chick (Kutchai & Steen, 1971).

The eggshell and membranes of *E. macquarii* appear to be dried regionally by embryonic influence, whereas they are dried over the whole surface simultaneously in avian eggs, through osmotic differences between the fluid in the membrane and the albumen (Kutchai & Steen, 1971; Lomholt, 1976; Tullett & Board, 1976). The reason for this difference may lie in the non-cleidoic nature of the former. It was shown that the eggs of *E. macquarii* imbibe liquid water under favourable conditions (Chapter 6). By drying the pores in one region at a time those elsewhere on the shell will presumably still contain water and allow liquid water flow. An "airlock" in a pore, such as in a chicken egg, would normally prevent the flow of liquid water.

Opaque shell, although drier than translucent shell, still contains

water (Table 5.5) resulting in about 6% of gaseous diffusion occurring in solution, the rest occurring by diffusion through air-filled spaces. The drying of the shell and membranes in chicken eggs is also incomplete (Kutchai & Steen, 1971; Lomholt, 1976) and the conductance increases as hydration decreases (Romijn, 1950; Lomholt, 1976).

In infertile eggs (which do not develop white patches) of *Crocodylus acutus* the G_{O_2} of the shell and membranes is five times higher when 30% of the water was removed (Lutz *et al.*, 1980). This reduction in water content is similar to that observed in opaque and translucent shell in *E. macquarii* (Table 5.5) and, as *C. acutus* probably forms a white patch as reported in other crocodilians (Webb *et al.*, 1977; Ferguson, 1982), suggests that the white patch in the eggs of crocodilians may serve the same function as in *E. macquarii*.

5.4.4 Rate of Loss of Water Vapour

Water was lost rapidly from eggs during the first four hours of being placed over silica gel, and then at a constant lower rate (Fig. 5.8). A similar phenomenon occurs in the eggs of *Chrysemys picta* (Tracy *et al.*, 1978) and birds (Hoyt *et al.*, 1979). The initial weight loss was assumed to be due to the evaporation of water from the surface and pores of the eggshell (Tracy *et al.*, 1978). As such, it does not represent shell conductance but is a combination of conductance and evaporation of superficial water. Thus the regression equations have a positive y-intercept (Table 5.2). The y-intercept is therefore a measure of the amount of this superficial water. For an egg of mean weight (10.423 g), the y-intercept was 0.188 g for day 1 eggs, 0.181 g for day 15 eggs, 0.177 g for day 30 eggs and 0.121 g for day 57 eggs, indicating a small decrease between each trial. The day 45 trial did not fit the pattern, losing

only 0.045 g in superficial water.

The conductance of the shell to water vapour increased throughout the incubation in all except the day 45 trial, which lost water more slowly than the day 1 trial ($0.02<p<0.05$). It is tempting to explain this result in terms of small sample sizes and large amounts of variation but the eggs used in the day 45 trial are from the same clutch as those in the day 15 and day 30 trials (Table 5.1) both of which fit the pattern. Hence I am unable to explain the result. The time of embryonic death, which was not known, probably occurred at different times in different eggs, because of their wide differences in conductance. Because no change in the rate of weight loss was noted between four hours and the asymptote in the rate of weight loss (Fig. 5.9) it was assumed that metabolic heating had little effect on the rate of water loss.

5.4.5 Functional Pore Area and Number of Pores

The estimate of the functional pore area in an egg of *E. macquarii* (0.0436% of the total shell area) is close to that of *Trionyx spiniferus* (0.052%, G. Packard *et al.*, 1979b). The pore length and egg size of *E. macquarii* (0.173 mm (Table 3.9) and 10.423 g (Table 3.2)) and *T. spiniferus* (0.172 mm and 10.15 g) are also similar. The functional pore area of the eggs of *Alligator mississippiensis* is 0.107% (G. Packard *et al.*, 1979b) and of avian eggs is 0.005 - 0.034% (Wangensteen *et al.*, 1970/71; Rahn *et al.*, 1976).

Counts of the pore number in *E. macquarii* (Chapter 3) ranges widely from 103 to 17,720 ($\bar{x}$ = 3,673 ± 4,599; n = 13). The variation in the conductance of the eggshell is not as large as the variation in pore number, but, eggs with fewer pores have larger pores and those with more

pores have smaller ones. The estimate of pore number based on functional pore area (3,500) is close to the mean estimate based on counts (3,673). The number of pores estimated in a mean egg of *E. macquarii* (10.423 g), by the equation of Tullett and Board (1977), is 3,878 which is close to the mean number observed.

6

WATER RELATIONS

6.1 INTRODUCTION

The shelled eggs of reptiles and birds isolate, to some extent, the developing embryos from the external environment. Needham (1931) coined the term "cleidoic" to describe eggs in which exchanges between the egg and the outside environment occur only in the gaseous state, under the normal conditions experienced during incubation. Most avian eggs are cleidoic (Needham, 1931). The non-cleidoic eggs, which include those of lepidosaurian reptiles, with the possible exception of some lizards (G. Packard *et al.*, 1977a), also exchange liquid water with the environment.

Eggs of the Chelonia range from soft-shelled to hard-shelled (Ewert, 1979). The ability of the soft-shelled chelonian eggs to absorb water and swell is well known (G. Packard *et al.*, 1977a), and may be advantageous to some species by enhancing the ability of the developing embryo to utilize yolk reserves and thereby grow larger before hatching (Tracy *et al.*, 1978; G. Packard *et al.*, 1980, 1981a,b; M. Packard *et al.*, 1982a).

The soft-shelled eggs of reptiles, although differing in structure between taxa, behave similarly with respect to water, i.e. the shell is able to expand to accomodate absorbed water (Needham, 1931, 1942; Cunningham & Hurwitz, 1936; Cunningham & Huene, 1938; Lynn & von Brand, 1945; Clark, 1946, 1953a,b; Legler, 1960; Dmi'el, 1967; Whitaker, 1968; Ernst, 1971a; Goel, 1976; Muth, 1977, 1980, 1981a; Tracy *et al.*, 1978; G. Packard *et al.*, 1981a,b). Indeed, some species may rely on this as a water supply for the embryo (Bustard, 1966; Tracy, 1980; Andrews & Sexton, 1981; M. Packard *et al.*, 1980). In contrast, hard-shelled eggs do not expand unless the shell cracks.

Water exchange, incubation time and hatchling weight appear to vary

according to the degree of calcification of the shell (M. Packard *et al.*, 1982a). Hence the soft-shelled eggs of squamates exchange water more rapidly and to a greater extent than the hard-shelled eggs of crocodiles and some chelonians and lizards, in response to the state of hydration of the incubation medium.

If incubated with half their surfaces in contact with substrates of different water potential or suspended on racks above the same substrates the soft-shelled eggs of *Chrysemys picta* (Tracy *et al.*, 1978; G. Packard *et al.*, 1981a) and *Chelydra serpentina* (G. Packard *et al.*, 1980, 1981b,d) prove very sensitive to water potential. Eggs on dry substrates lose more water, or gain less water, than those on wetter substrates; those suspended above the driest substrates lose more water than those above wetter ones. The hatchlings are larger and hatch later in wetter conditions, but water potential does not influence the hatching success rate. In contrast, the hard-shelled eggs of *Trionyx spiniferus* are relatively insensitive to substrate water potential, and all eggs sustain a net weight loss before hatching (G. Packard *et al.*, 1979a, 1981c). Hatching success and the weight of hatchlings are not influenced by substrate water potential. The hard, expansible-shelled eggs of *Emydoidea blandingii* respond to substrate water potential in an intermediate manner (G. Packard *et al.*, 1982). Weight changes in the eggs during incubation are strongly influenced by substrate water potential, but although the eggs that lose the least water give rise to the largest hatchlings, the association of hatchlings size and net changes in egg mass is equivocal. Hatching success appears to be influenced only slightly by substrate water potential.

The soft-shelled eggs of the lizards (*Sceloporus undulatus* (Tracy, 1980) and *Callisaurus draconoides* (M. Packard *et al.*, 1980)) tolerate a

relatively narrow range of water potentials (-200 to -590 kPa). They certainly incorporate water from the incubation medium into the developing embryo, because the hatchlings are heavier than the eggs at oviposition. However, in *C. draconoides* but not *S. undulatus* the size of hatchlings is related to the water potential of the substrate. This is in contrast to the soft-shelled eggs of *Chrysemys picta* for which heavier hatchlings arise from eggs that absorb more water but the hatchlings weigh less than the fresh eggs (Tracy *et al.*, 1978; G. Packard *et al.*, 1981a). It is not known whether the absorbed water is incorporated into the embryo or whether it merely allows the water contained within the egg at laying to become available for incorporation into the embryo.

The strategy of laying eggs that must imbibe water to complete development should be advantageous to species that live in an environment where water may be limited to the female (unlike freshwater chelonians). The sensitivity of soft-shelled eggs to the water conditions of the environment varies with the structure of the shell. The lizard, *Anolis auratus*, which lays eggs in a drier environment, has an eggshell that is thicker, with a denser fibrous layer and thicker calcified matrix, loses water at a slower rate than *A. limifrons* from a moister environment (Andrews & Sexton, 1981).

The hard, expansible-shelled eggs described by Ewert (1979) have brittle shells when laid but behave like soft-shelled eggs during incubation when they swell and the shell cracks. Eggs of *Chelodina longicollis* have been described as intermediate between hard-shelled and hard, expansible-shelled because the mineral layer begins to fracture into small flakes midway through incubation (Ewert, 1979). The eggs of *C. longicollis* that I incubated appeared to behave in a similar fashion to those of *Emydura macquarii*, although I did not observe them to flake.

Hence an investigation of the eggs of *E. macquarii* is significant as they may represent an intermediate between the hard-shelled and hard, expansible-shelled eggs studied to date.

I examined the effect of the degree of contact of an egg with substrates of various water potentials on the weight changes of the egg throughout the incubation, on the hatching rate and on the weight of the hatchlings. In the completed nest, eggs of *E. macquarii*, like most other chelonians (Cagle, 1950; Ackerman, 1977; G. Packard *et al.*, 1977a, 1979a, 1981c), are contained in a chamber usually free of soil. Only some eggs are in contact with the walls of the chamber, the rest are supported in the nest by other eggs. Although the eggs are non-cleidoic only those in contact with the walls of the nest chamber are potentially able to absorb water directly from the substrate.

I examined the possibility that water may flow between eggs along fluid bridges set up when the eggs are laid and their moist surfaces contact each other, because it has been assumed that eggs incubated on racks above substrates simulate eggs held away from the walls of the nest chamber by other eggs (G. Packard *et al.*, 1980, 1981b,c, 1982).

G. Packard *et al.* (1977a) argued that many reptilian eggs, both soft-shelled and hard-shelled, absorb liquid water from the incubation substrate and lose water vapour to the nest atmosphere. Tracy *et al.* (1978) formalized this with the equation

$$\dot{m}_{st} = \dot{m}_s - \dot{m}_a \qquad (6.1)$$

where $\dot{m}_{st}$ = net rate of water exchange of an egg (g. min^{-1})

$\dot{m}_s$ = rate of water uptake from the substrate (g. min^{-1})

$\dot{m}_a$ = rate of water vapour loss to the nest atmosphere (g. min^{-1}).

They measured $\dot{m}_{st}$ and $\dot{m}_a$ for eggs with half their surface contacting the

substrate and calculated $\dot{m}_S$. To test the validity of their method I measured $\dot{m}_{st}$ and $\dot{m}_a$ using the same method employed by Tracy *et al.* (1978), and also measured $\dot{m}_S$ directly for comparison.

To test the idea that female chelonians may select the substrate type in which to lay eggs to suit the conditions of soil water necessary for successful incubation (e.g. Stancyk & Ross, 1978), I collected soil samples from natural nests at Lake Bonney and prepared curves relating their soil water content to water potential. In this way I could detect any selection of soil within a narrow range of water potential characteristics by nesting *E. macquarii*. Selection of nest sites by females may be an important adaptation because substrate water potential and conductivity are determinants of water balance in incubating eggs (Tracy *et al.*, 1978). *Trionyx spiniferus*, which lays hard-shelled eggs, usually selects a course sand or fine gravel (G. Packard *et al.*, 1979a).

All chelonians dig the nest chamber with the hind limbs in a stereotyped manner (Ehrenfeld, 1979; Ackerman, 1980). Nest depth is generally positively correlated with size of female because it is limited by the length of the hind limbs (Hendrickson, 1958; Carr, 1967; Mahmoud, 1968; Ernst, 1970; Bustard, 1972). However, within some species there is not a positive correlation between nest depth and female size (e.g. *Malaclemys terrapin*, Montevecchi & Burger, 1975) and others increase nest depth by constructing a "body pit" first (e.g. *Chelonia mydas* and other marine turtles, Bustard, 1972). The shapes of chelonian nests, which have been described as pear-shaped (Bustard, 1972), jug-shaped (Cagle, 1937, 1950), flask-shaped (Mahmoud, 1968; Ernst, 1970; Moll & Legler, 1971; Ehrenfeld, 1979; Alho & Pádua, 1982) and goblet-shaped (Mahmoud, 1968), are also similar. These names indicate that the nest consists of a narrow entrance leading to an expanded egg chamber. The entrance is plugged with soil

after oviposition. The depth of soil between the eggs and the surface has important influences on the developing embryos. For example, diurnal temperature fluctuations decrease with depth which may influence the synchronization of hatching (Chapter 4) and the sex of the hatchlings (Chapter 7). Therefore the depth and shape of nests of *E. macquarii* were examined and compared to other species.

6.2 MATERIALS AND METHODS

6.2.1 Field Samples

Samples of soil were taken at nest depth throughout the incubation period, within 0.5 m of seven natural nests and two nests at the tortoise farm. Samples were sealed in preweighed glass jars with metal lids containing waxed cardboard inserts. On return to the laboratory each sample was weighed, dried to constant weight at 105°C, reweighed and the water content calculated.

Samples chosen to represent soil types used by *E. macquarii* in the construction of nests were taken from nine nests on the shores of Lake Bonney. Curves relating water potential to percent water content of all nine samples were determined by ceramic plate extraction at 0.01, 0.1, 1.0 and 15.0 bars (1 bar = 100 kPa), by courtesy of CSIRO Division of Soils.

The dimensions of thirteen nests that had been robbed by predators were measured with a steel tape measure at Lake Bonney, Barmera on 9/4/78. The maximum and minimum diameters of the oval-shaped nest entrance, the depth from soil surface to bottom of nest and maximum length of the bottom of the nest chamber were measured. Predators appeared to expose the egg chamber by digging out the nest plug and remove the eggs without further digging. If the original nest entrance had been widened, as indicated by the claw marks of the predator, the nest was disregarded. Measurements made on predated nests were assumed to be similar to those of intact nests.

6.2.2 Artificial Incubation

Trials were conducted to test the effect of substrate water potential

on the state of hydration of the eggs and hatchlings, and the hatching success rate of *E. macquarii*. The sand used as an incubation substrate was taken from a nesting beach at Lake Bonney, sieved to exclude any stones and wood and heat sterilized. Eggs were from natural nests at Lake Bonney.

An initial trial consisted of four groups of five eggs from one clutch buried in substrates with water potentials of -110, -290, -630 and -1,160 kPa and incubated at 25°C. As this range of water potentials was insufficient to stress the eggs, as indicated by their hatching success rate, a second trial, consisting of groups of eight eggs buried in substrates of -50, -710, -2,140 and -3,550 kPa was set up. Each group was duplicated five times, one group at each of the temperatures 25, 26, 28, 30 and 32°C (see Chapter 7). Nine clutches of eggs were involved. Only one group of eight contained two eggs from one clutch, and all groups contained one egg from each of six clutches.

A third trial was set up with three groups of eggs: one buried in the substrate, one placed with half their surface in contact with the substrate and one suspended above the substrate on racks made of plastic mesh. Water potentials of the substrates were -220, -710, -1,140, -2,140 and -3,550 kPa and the eggs were incubated at 30°C. The number of eggs used in each group is given in Table 6.1.

The water potentials used include the range measured in the field in previous incubation seasons. After the incubation, a water potential curve was constructed using thermocouple psychrometry with a Wescor Inc. HR-33T Dew Point Microvoltmeter so that the data were comparable with other studies (e.g. Tracy *et al.*, 1978; G. Packard *et al.*, 1979a, 1980, 1981a,b,c, 1982; M. Packard *et al.*, 1980). This was necessary because

TABLE 6.1 NUMBER OF EGGS IN EACH GROUP IN THE THIRD WATER POTENTIAL TRIAL

The number of clutches from which eggs were drawn are in brackets

	Water Potential (kPa)				
Position	-220	-710	-1,140	-2,140	-3,550
Buried	8 (6)	7 (3)	10 (3)	9 (6)	8 (6)
Surface	9 (6)	6 (3)	10 (3)	9 (6)	9 (6)
Rack	8 (6)	10 (3)	9 (2)	9 (6)	8 (6)

the results of the ceramic plate extraction and thermocouple psychrometry were not identical. As the water potential of the substrate containing 0.50% water could not be measured using thermocouple psychrometry it was estimated from the regressions describing weight changes in eggs buried in and sitting on substrates as a function of water potential (Figs. 6.10, 6.11). This yielded estimates of -3,400 and -3,700 kPa so the water potential of the incubation substrate containing 0.50% water was assumed to be -3,550 kPa (mean). Estimates were not calculated from regressions relating weight loss to substrate water potential and relative humidity for eggs suspended above the substrate because of the lower significance (Table 6.5).

The vapour pressure of the atmosphere in the incubation chamber was assumed to come to equilibrium within 30 minutes of the chamber being closed and the relative humidity in the chamber was calculated from the equation

$$\Psi = R.T.\ln {}^{\rho_a}/_{\rho_s} \qquad (6.2)$$

where Ψ is the water potential of liquid in the substrate (bars), R is the gas constant of water vapour (4.62 bars.K^{-1}), T is the absolute temperature (K) and ρ_a and ρ_s are the vapour densities of the air and of

air saturated with water vapour at the same temperature respectively (Tracy *et al.*, 1978).

To maintain the water potential in each trial the incubation chamber, with eggs in place, was weighed daily to 0.01 g and distilled water added to compensate for any weight losses. Each egg was weighed periodically to the nearest milligram on a Sartorius electronic pan balance, and the amount of distilled water added to the incubation chamber was adjusted to compensate for any changes in the weight of the eggs. When eggs were removed for weighing the incubation substrate was stirred to ensure homogeneous distribution of water.

As the respiratory substrate of eggs is lipid, which has an R.Q. of about 0.7 (Ackerman, 1977), equal masses of CO_2 and O_2 are exchanged during incubation (G. Packard *et al.*, 1979a). Therefore changes in egg mass during incubation may be attributed to water exchange.

Each egg in the final trial was covered with a cage of plastic mesh just before hatching so that egg and hatchling could be matched. Hatchlings were brushed free of adhering sand or shell fragments and weighed to the nearest milligram within six hours of hatching. They were killed by freezing and then dried to constant mass over silica gel under vacuum at room temperature, reweighed and their water content calculated. Although hatchling fresh mass was used here as an index of hatchling size, a size index based on linear characteristics such as carapace length and width and head width (as well as the live and dry masses) may be a more powerful index (G. Packard *et al.*, 1981a). Unfortunately the hatchlings of *E. macquarii* had been desiccated and many of the linear dimensions distorted when this method was published.

The albumen and yolk from 53 eggs used in the O_2 and CO_2 conductance experiments (Chapter 5) were collected and the water potential measured in a Wescor Inc. 5100C vapour pressure osmometer. These eggs were incubated sitting on substrates of -1,140 kPa at 30°C. As it was difficult to remove the egg contents without rupturing the yolk (vitelline) membrane, 50 measurements represent mixed yolk and albumen. In three eggs, including one on day 1, the yolk and albumen were measured separately. No measurements were taken beyond day 30 of the incubation after which the embryo and allantois are the main contents of the eggs.

6.2.3 Pyramid Experiment

To test for water flow between eggs via fluid bridges, pyramids consisting of a triangle of eggs half buried in the incubation substrate with a fourth egg sitting on top were constructed. The eggs were obtained from tortoises with oxytocin. When laid they were weighed and placed in the trial immediately, while the shell was still wet with fluid from the female. This was done to simulate the situation in a natural nest where eggs are laid within a relatively short period of time and fluid bridges could form between eggs.

An incubation substrate with a water potential of -710 kPa was chosen because, over the course of the incubation period, eggs sitting on such substrates gain weight (Fig. 6.7), but the hatching rate is not affected (Table 6.2). The water in the substrate contained 0.10% fluoroscein by weight. Each egg was assumed to contain 7.5 ml of water. One microcurie (μC) of tritium was added to the incubation chambers for each estimated 100 ml of water in the chamber, including that in the egg. The water content of the substrate matched the estimated water content of the eggs. As fluoroscein is soluble in water and since as little as one part in

forty million parts of water is visible under ultra-violet light (Gurr, 1971), its presence would indicate whether liquid water had flowed from the substrate into the eggs. Medical applications of fluoroscein suggest that it is non-toxic (Gurr, 1960, 1971). The tritium, expressed as specific activity ($\mu C.\ell^{-1}$), would show how much total water exchange had occurred. Figure 6.1 illustrates the way the eggs were set up and the expected paths of water exchange. The incubation chambers were glass desiccators sealed to the atmosphere. Respiration is so low at the beginning of the incubation period (Chapter 4) that no significant changes in gas concentrations were expected in the chamber during the experiment. Control pyramids in which the eggs contacting the substrate were replaced with large glass marbles were also set up. Each marble was ringed with petroleum jelly around the equator. The jelly touched neither the substrate nor the control egg on the marbles and prevented liquid water flow over the surface of the marble from the substrate to the control egg. Hence water exchange between the control egg and the surrounding atmosphere could occur only in the gas phase (Fig. 6.2). By subtracting the specific activity of the control egg from that of the trial eggs any liquid water flow from the substrate through or around the eggs contacting the substrate to the trial egg could be detected.

Initial runs in which eggs were left for 0.5, 2, 9, 18, 30 and 48 hours were made to establish the most appropriate time for the experimental run. Each run comprised one pyramid of eggs and one control pyramid (total = 30 eggs). Forty-eight hours was chosen as the most appropriate time to run the experiment because there was a relatively large difference in specific activity between the trial eggs and the eggs on the substrate but the specific activity in all eggs was below the equilibrium (i.e. when the tritium was distributed evenly throughout the chamber).

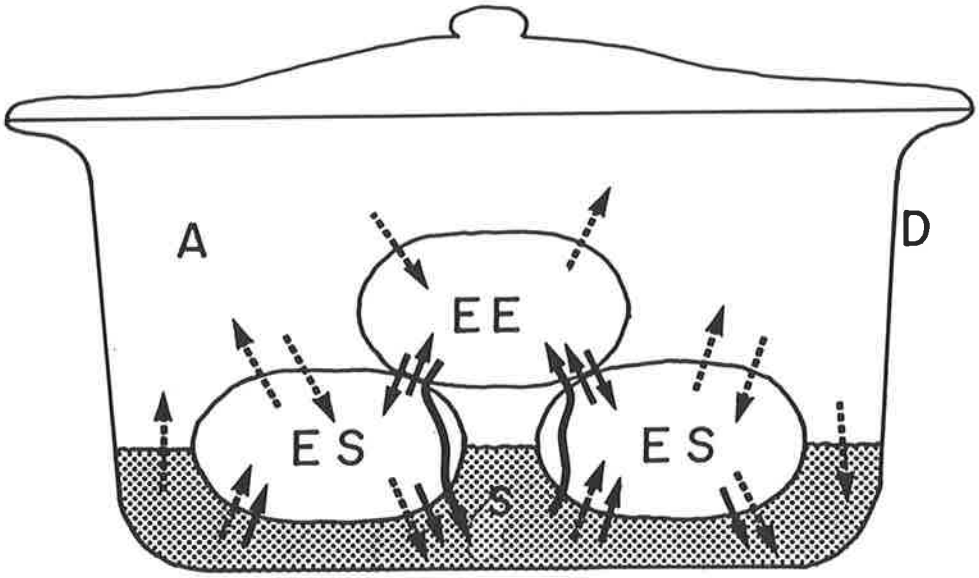


FIGURE 6.1

SKETCH OF PYRAMID EXPERIMENT.
ES represents eggs contacting the substrate, S.
EE represents the egg suspended in the air, A, above the incubation substrate by ES. Sealed glass desiccators, D, were used as experimental chambers.
Arrows represent possible avenues of water flow in the liquid (⟶) and gas (⇢) phases.

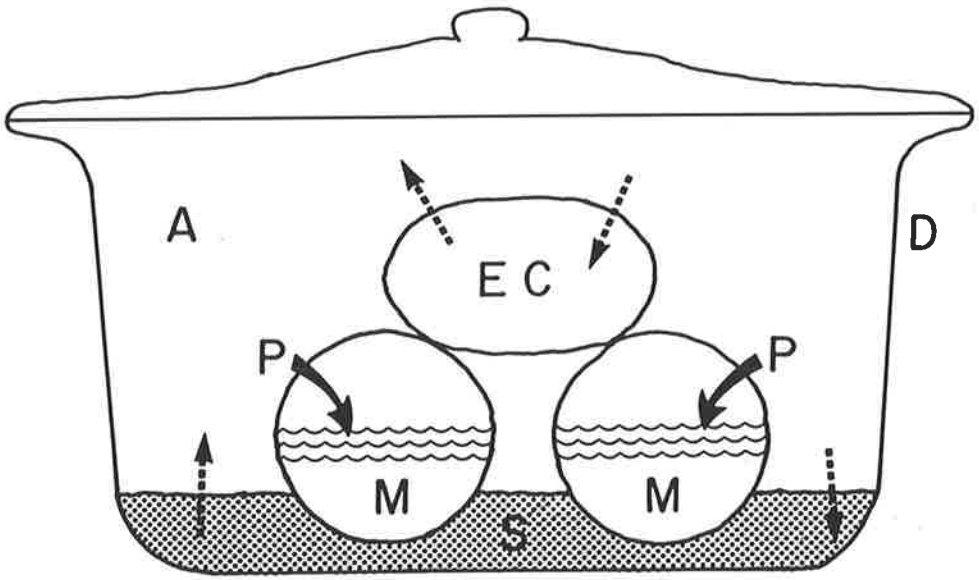


FIGURE 6.2

SKETCH OF THE CONTROL IN THE PYRAMID EXPERIMENT.
M represents large glass marbles and P is a thin layer of petroleum jelly that prevented flow of liquid water around the face of the marble to the control egg on top (EC); see Fig. 6.1 for full explanation of symbols.

The experiment used 26 eggs contacting the substrate, 10 trial eggs on top of these and 10 control eggs in three chambers. The eggs were from five clutches. Some "pyramids" were constructed of two eggs sitting on five eggs, reducing the number of eggs contacting the substrate from 30 to 26.

At the end of the experiment eggs were cut open with scissors and an edge cutting disc in a dental drill, and the yolk, albumen and shell fractions separated for viewing under ultra-violet light. Some eggs were weighed at the end of the experiment before being opened.

After separation of egg fractions, albumen and eggshell were placed under ultra-violet light for detection of fluoroscein. The yolk itself was found to fluoresce slightly under ultra-violet light so was not examined for fluoroscein. The outside and inside surfaces of all eggshells were examined carefully under ultra-violet light, especially at the point of contact between eggs.

After the samples had been examined for fluoroscein they were frozen. Water was extracted from the albumen fraction by vacuum sublimation for measurement of tritium content (specific activity). Half a millilitre of water was mixed with 8 ml of scintellation fluid and counted in a Packard TRI-CARB 3002 scintellation spectrometer. The scintellation fluid contained toluene (375 ml.), dioxan (375 ml.), ethanol (250 ml.), napthalene (80 g) and P.P.O. (5 g).

There were large variations in the specific activity of eggs from different clutches so the eggshell thickness of all eggs was measured with a screw micrometer as described in Chapter 3. As conductance is inversely proportional to shell thickness (Ar *et al.*, 1974) the specific

activity of each egg was multiplied by the thickness of the eggshell from the middle of the egg and divided by the overall mean middle thickness of the eggs used in the experiment (= 0.188 mm.) i.e. RSA = MET . ASA . 1/0.188 where RSA = relative specific activity ($\mu C \cdot l^{-1}$), MET = middle eggshell thickness (mm.), ASA = actual specific activity ($\mu C \cdot l^{-1}$). This procedure minimised differences in counts of specific activity due to differences in shell thickness between eggs, making the data comparable.

6.3 RESULTS

6.3.1 Field Samples

Tortoises construct their nests in soils with a wide range of water potential characteristics (Fig. 6.3). The samples range from heavy clay to a mixture of coarse sand and shell grit and represent the extremes of soil types found around the lake. The higher proportion of eggs in sandy soils reflects the relative abundance of this soil type over that of clay soils. The water potential curve for soil in which eggs were incubated in the laboratory is included in Figure 6.3.

All nests for which water content was measured throughout the incubation (Fig. 6.4) had a normal hatching success rate. These nests were deposited within the range of soil types indicated in Figure 6.3, which suggests that *E. macquarii* does not choose its nesting site on the basis of water potential characteristics. Hatching success was not influenced by the soil type in which the nest was deposited.

The method of construction and shape of the nests of *E. macquarii* are similar to those of other species of chelonians; a narrow shaft leading to an expanded egg chamber dug with the hind feet (Goode, 1965). The egg chamber is extended an average of 11 mm in one direction, apparently backwards from the position of the tortoise. Mean dimensions of nests measured at Lake Bonney, were calculated (Table 6.2).

I saw only one female laying eggs. The eggs were not manipulated and fell onto other eggs already in the chamber. In many nests one or more eggs had depressed fractures where the end of one egg released by the female cracked another. The underlying shell membranes remained intact and such cracks did not affect the chance of the egg hatching.

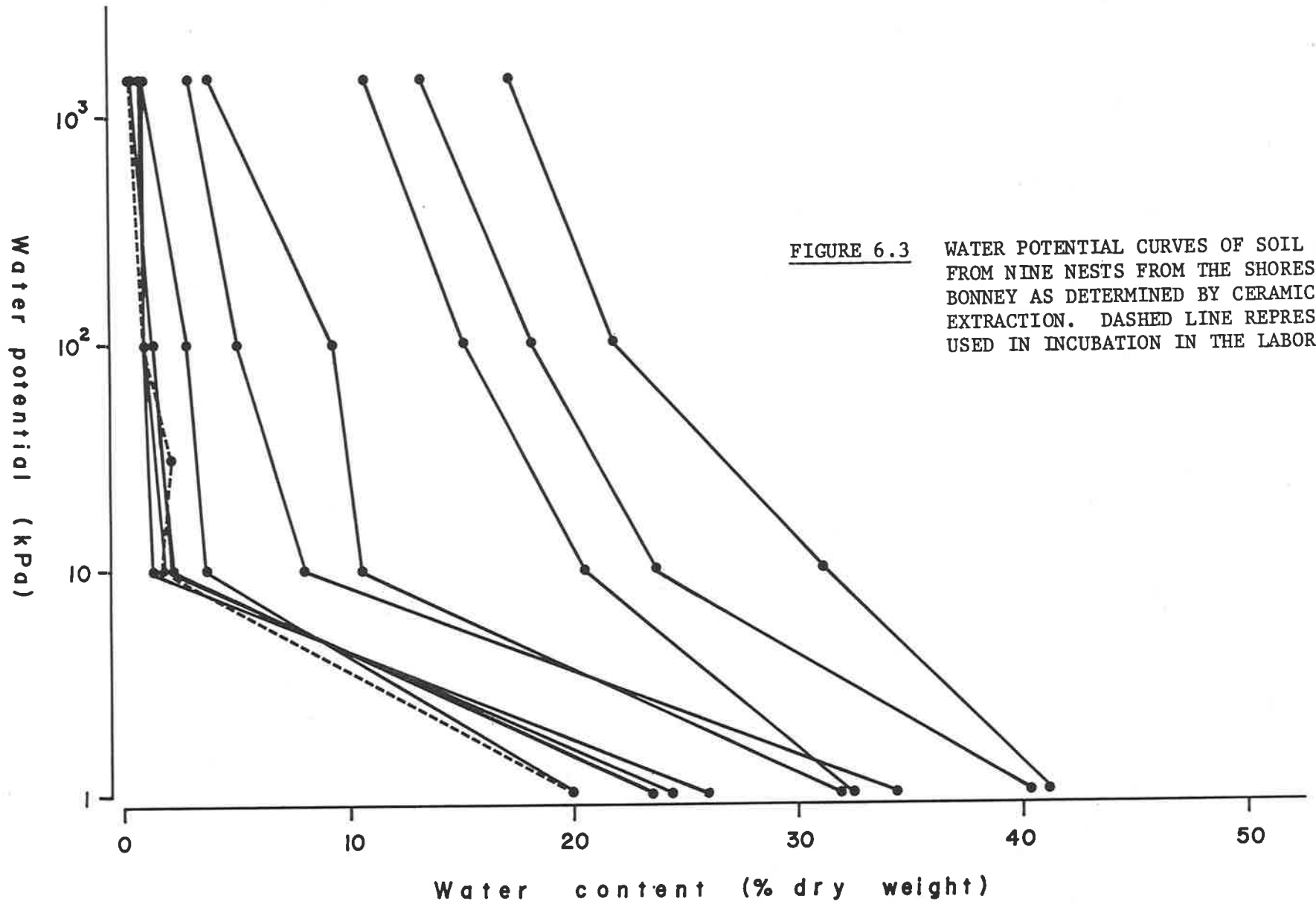


FIGURE 6.3 WATER POTENTIAL CURVES OF SOIL SAMPLES FROM NINE NESTS FROM THE SHORES OF LAKE BONNEY AS DETERMINED BY CERAMIC PLATE EXTRACTION. DASHED LINE REPRESENTS SOIL USED IN INCUBATION IN THE LABORATORY.

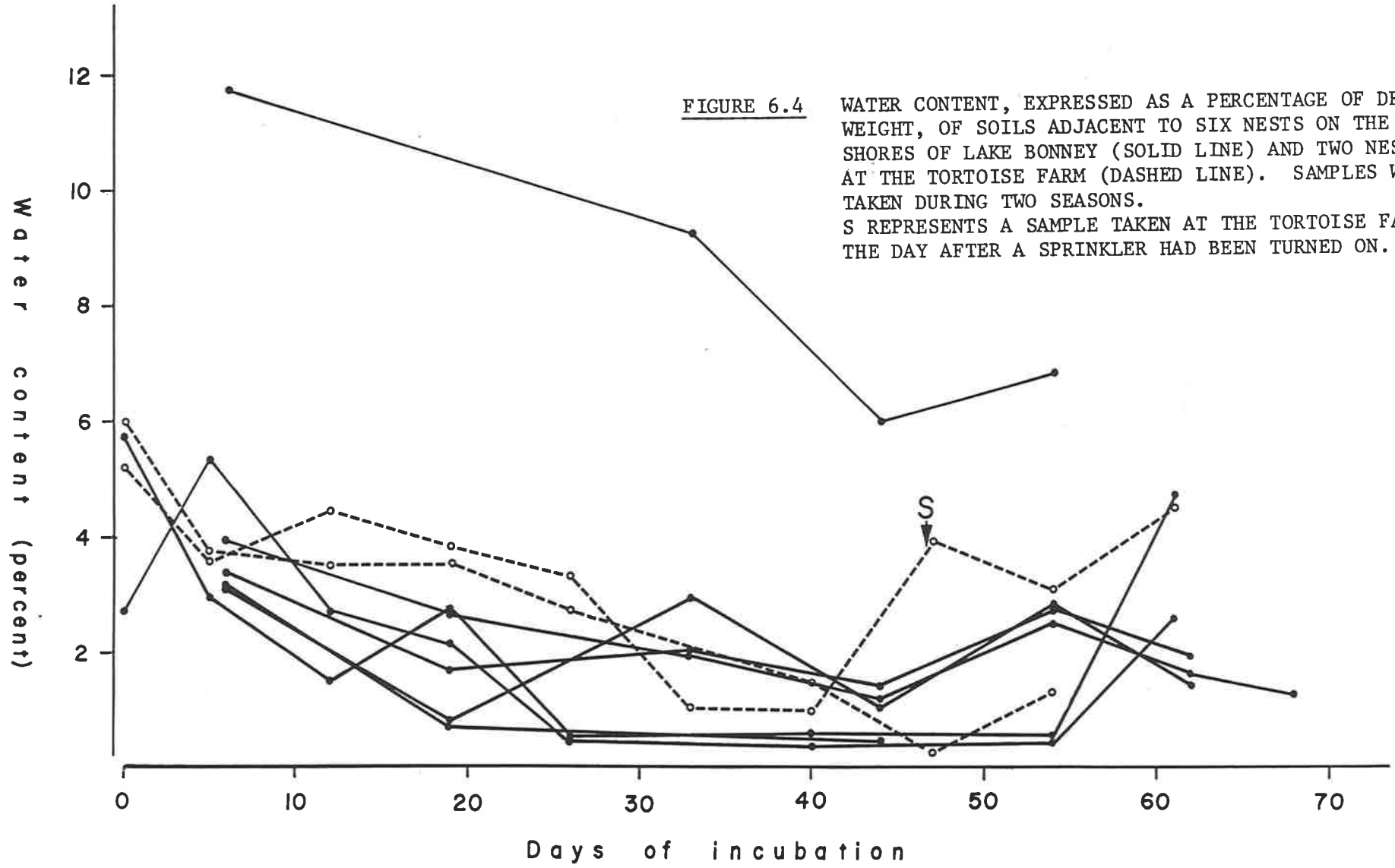


FIGURE 6.4 WATER CONTENT, EXPRESSED AS A PERCENTAGE OF DRY WEIGHT, OF SOILS ADJACENT TO SIX NESTS ON THE SHORES OF LAKE BONNEY (SOLID LINE) AND TWO NESTS AT THE TORTOISE FARM (DASHED LINE). SAMPLES WERE TAKEN DURING TWO SEASONS.
S REPRESENTS A SAMPLE TAKEN AT THE TORTOISE FARM THE DAY AFTER A SPRINKLER HAD BEEN TURNED ON.

As in other species the nest plug is placed by the tortoise with very little, if any, of the substrate falling between the eggs. Some nests in very sandy soil suffered some collapse of sand into the egg chamber but the effect this had on the hatching rate is not known.

TABLE 6.2 DIMENSIONS OF PREDATED NESTS (cm) MEASURED AT LAKE BONNEY ON 9/4/78

Measurement	Diameter of Entrance		Depth	Length of Expanded Egg Chamber
	Maximum	Minimum		
Mean	10.0	8.1	16.7	11.1
S.D.	1.6	1.5	1.9	2.5
Range	8.5 - 14.0	6.0 - 10.5	15.5 - 19.5	9.5 - 18.0

6.3.2 Artificial Incubation

The sand used to incubate eggs in the laboratory (Fig. 6.5), was typical of the more common substrates used by nesting tortoises at Lake Bonney. Soil water contents ranging from 0.50 - 21.95% were used to incubate the eggs in the laboratory, as eggs in the field mostly experience water potentials within this range (Fig. 6.4). Water contents lower than 0.50% are very difficult to maintain at a constant level so soils drier than this were not tested.

Hatching Success at Different Water Potentials

Eggs buried in substrates of -50 kPa, which contains slightly more water than the field capacity of the substrate (20 - 21%), die within a

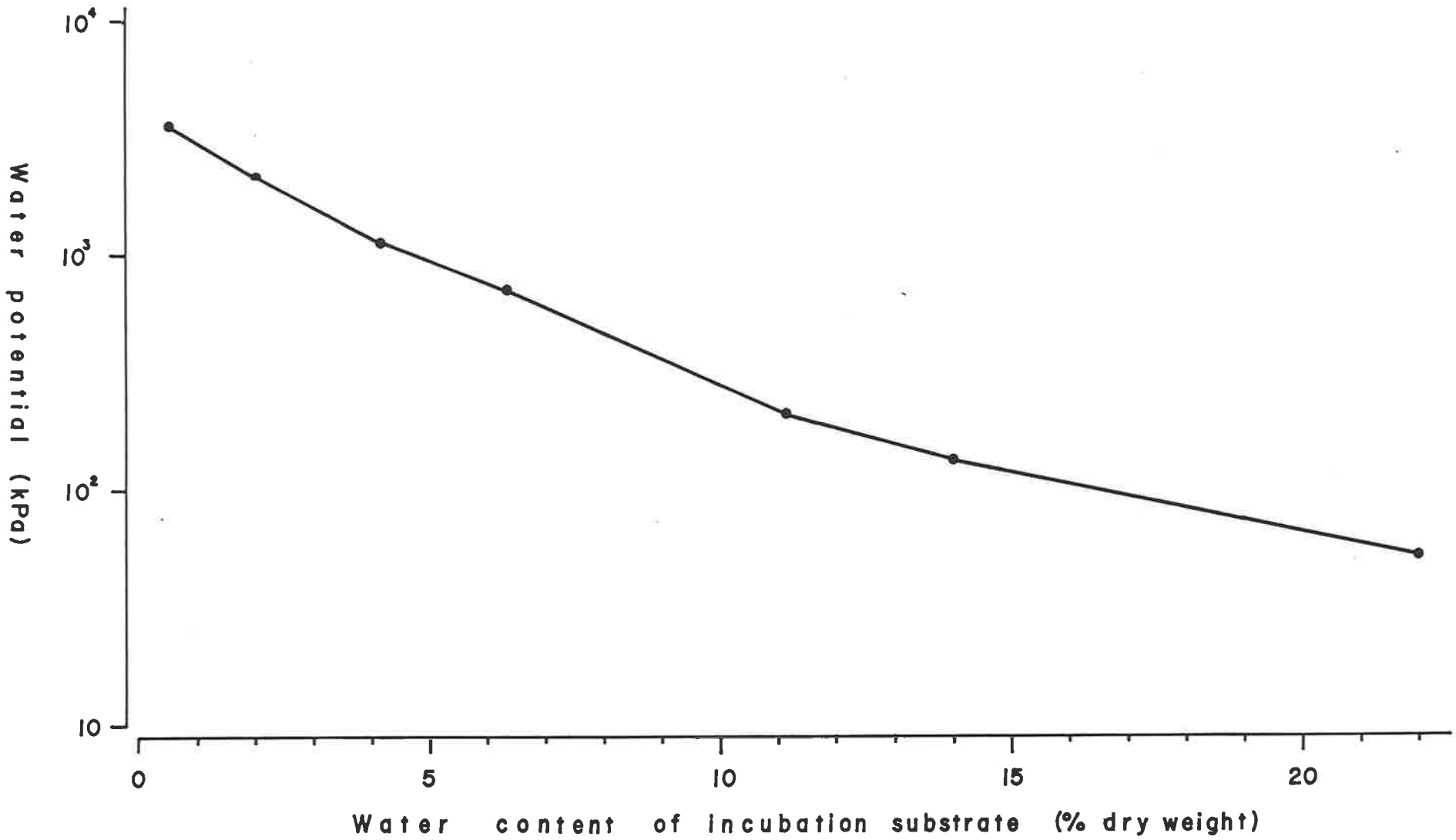


FIGURE 6.5 CURVE RELATING WATER POTENTIAL (kPa) TO PERCENT WATER CONTENT OF THE INCUBATION SUBSTRATE USED IN THE LABORATORY DETERMINED USING THERMOCOUPLE PSYCHROMETRY. WATER POTENTIAL AT 0.5% WATER WAS CALCULATED BY EXTRAPOLATION (see Section 6.3.2).

few days. At -110 and -220 kPa, which are near to field capacity, the hatch rate is diminished (30.8%, n = 13). At these water potentials eggs die at varying stages of incubation from very early until just before hatching. At all other water potentials good hatching success rates (71.4 - 100%) occur in buried eggs (Table 6.3). The determinant of hatching success appears to be water potential and not incubation temperature between 25 and 32°C.

For eggs with half their surface contacting the substrate the hatching rate is depressed at -220 kPa (44.4%), but is still 3.6 times higher than eggs buried at the same potential. Eggs on the surface at all other water potentials have high hatching success rates (88.9 - 100%) (Table 6.3).

All eggs suspended on racks (n = 44) hatch successfully regardless of the water content of the substrate below.

Masses of Hatchlings Incubated at Different Water Potentials

A Kruskal-Wallis tests revealed no difference in the wet (Hc = 17.00, $0.25<p<0.50$) and dry (Hc = 11.29, $0.50<p<0.75$) masses of hatchlings as a percentage of fresh egg mass in each trial (Table 6.4). The variances in wet and dry masses are equal (Bartlett's test, $0.25<p<0.50$, Zar, 1974).

Changes in Mass of Eggs as a Function of Time

Only eggs that hatched are included in this analysis. All eggs, irrespective of the water content of the substrate or their position relative to the substrate, lose water for the first third of the incubation period (Figs. 6.6 - 6.8). The amount of water lost during, and the pattern of change after, this time are related to the water potential of the substrate and the position of the egg (Table 6.5, Figs. 6.6 - 6.8).

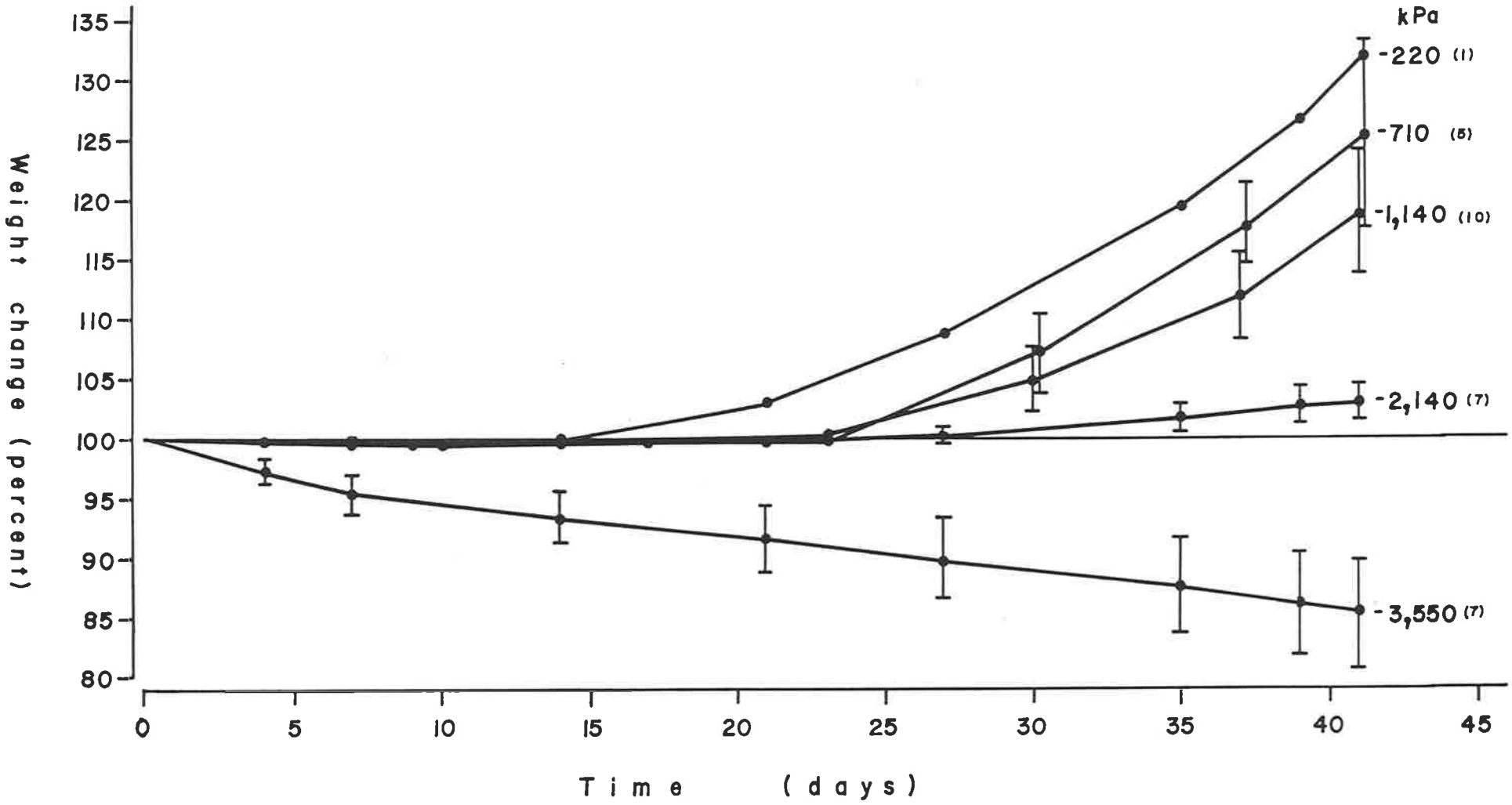


FIGURE 6.6 CHANGE IN WEIGHT OF EGGS BURIED IN SUBSTRATES WITH WATER CONTENTS OF -220, -710, -1,140, -2,140 AND -3,550 kPa. BARS REPRESENT ONE STANDARD DEVIATION. SAMPLE SIZES ARE IN BRACKETS.

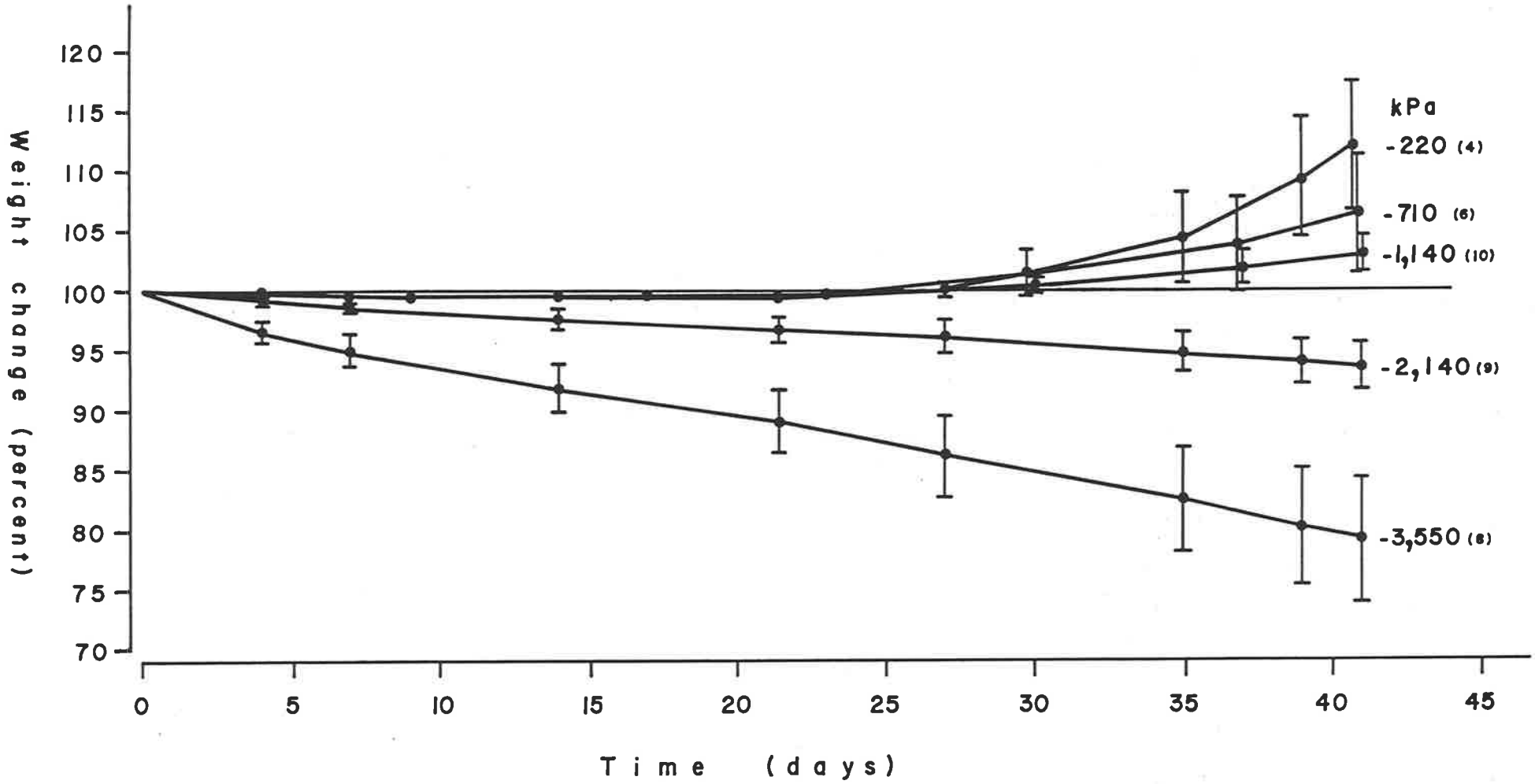


FIGURE 6.7 CHANGE IN WEIGHT OF EGGS ON SURFACE (50% OF SHELL AREA IN CONTACT WITH SUBSTRATE) OF SUBSTRATES OF VARIOUS WATER POTENTIALS. REST SAME AS IN FIG. 6.6.

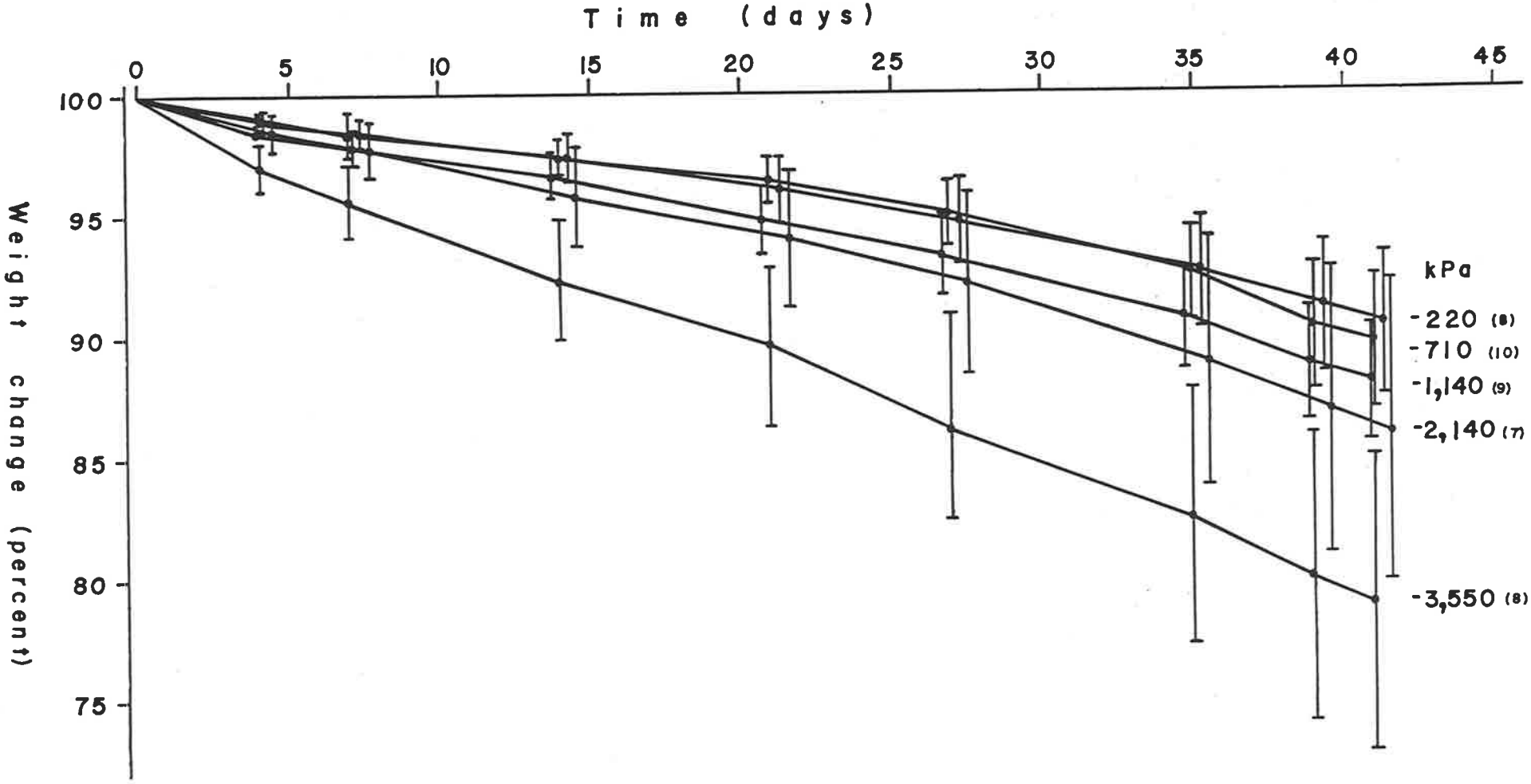


FIGURE 6.8 CHANGE IN WEIGHT OF EGGS SUSPENDED ON RACKS OVER SUBSTRATES OF VARIOUS WATER POTENTIALS. REST SAME AS FIG. 6.6.

TABLE 6.3 HATCHING SUCCESS RATES (PERCENT) WITH SUBSTRATES OF DIFFERENT WATER POTENTIALS. RELATIVE HUMIDITIES OVER SUBSTRATES OF GIVEN WATER POTENTIALS ARE ALSO GIVEN. SAMPLE SIZES IN BRACKETS.

Water Potential (kPa)	-50	-110	-220	-290	-630	-710	-1,140	-1,160	-2,140	-3,550
Relative Humidity (%)	99.96	99.92	99.84	99.79	99.55	99.49	99.19	99.18	98.48	97.50
Position -										
Buried	0 (40)	60 (5)	12.5 (8)	100 (5)	100 (5)	80.9 (47)	100 (10)	100 (5)	87.8 (49)	79.2 (48)
Surface			44.4 (9)			100 (6)	100 (10)		100 (9)	88.9 (9)
Rack			100 (8)			100 (10)	100 (9)		100 (9)	100 (8)

TABLE 6.4 MEAN FRESH EGG MASS (g), HATCHLING MASS (g) AND THE HATCHLING MASS EXPRESSED AS A PERCENTAGE OF FRESH EGG MASS, FOR EGGS INCUBATED IN MEDIA OF DIFFERENT WATER POTENTIALS AND IN DIFFERENT POSITIONS RELATIVE TO THE INCUBATION MEDIUM. A KRUSKAL-WALLIS TEST REVEALED NO SIGNIFICANT DIFFERENCE BETWEEN TRIALS IN THE WEIGHT OF HATCHLINGS AS A PERCENTAGE OF FRESH EGG WEIGHT (Hc = 17.00, $0.25<p<0.50$).

Trial	Buried					Surface					Suspended				
Ψ (kPa)	-220	-710	-1,140	-2,140	-3,550	-220	-710	-1,140	-2,140	-3,550	-220	-710	-1,140	-2,140	-3,550
$\bar{x}$ egg mass (g)	10.418	10.377	11.180	10.495	10.062	10.519	11.243	11.457	10.548	10.746	10.270	10.637	10.291	10.569	10.119
± S.D.	-	0.957	1.842	0.791	0.831	1.058	1.755	1.840	0.972	0.690	0.955	0.919	0.839	0.874	0.897
$\bar{x}$ hatchling mass (g)	5.281	4.779	5.166	5.137	4.985	5.360	5.247	5.304	5.160	4.985	5.009	5.202	4.916	5.093	4.821
± S.D.	-	0.424	0.570	0.387	0.365	0.350	0.698	0.582	0.244	0.365	0.332	0.489	0.351	0.381	0.457
$\bar{x}$ hatchling mass (%)	50.70	46.16	46.66	49.03	46.43	51.20	46.85	46.72	49.14	46.43	48.93	48.90	47.82	48.29	47.68
± S.D.	-	3.10	3.55	3.05	2.62	4.35	2.36	3.50	3.23	2.62	2.62	1.80	1.24	2.69	2.60
n	1	5	10	7	7	4	6	10	9	8	7	10	9	9	8

Buried Eggs Eggs incubated in the driest substrate initially lose water more rapidly than all other eggs and continued to lose water until hatching (Fig. 6.6). All eggs at other water potentials ultimately gain weight, the amount being dependent on the water content of the substrate. The eggs at -2,140 kPa only marginally gain water with a mean increase of only 2.9%. There is considerable overlap in the water gain of eggs buried in -220, -710 and -1,140 kPa with one egg at -710 kPa gaining 0.2% more water than the only egg to survive at -220 kPa. Of those eggs that survived more than half the incubation period in the -220 kPa trial the one that survived to hatching gained water at the slowest rate.

Eggs that gain weight crack to accomodate the extra water (Fig. 6.9). In eggs gaining the most water cracks in the calcareous shell become very wide, exposing the underlying shell membranes and shedding some of the hard shell.

Eggs on the Surface Only eggs incubated on the wettest substrates gain weight by the end of the incubation period (Fig. 6.7). However, the weight gain is considerably less (62 - 84% less) than that experienced by eggs buried in substrates containing the same amount of water (Table 6.5). Eggs on the driest (-3,550 kPa) substrate lose 27% more water than those buried in the same substrate. As with buried eggs there is considerable overlap between the weight gained by eggs incubated at -220, -710 and -1,140 kPa.

Eggs on Racks All eggs suspended above the substrate lose water throughout the entire incubation period (Fig. 6.8). Weight loss ranges from 6.2% for an egg over the -220 kPa substrate to 27.5% for one over -3,550 kPa.

FIGURE 6.9 AN EGG INCUBATED BURIED IN A SUBSTRATE OF -710 kPa. THE CRACKING OF THE CALCIUM CARBONATE SHELL ALLOWED THE EGG TO SWELL AND ACCOMODATE ABSORBED WATER.

TABLE 6.5 TOTAL CHANGE IN EGG WEIGHT (PERCENT) DURING INCUBATION IN RELATION TO POSITION RELATIVE TO SUBSTRATES OF DIFFERENT WATER POTENTIAL

Position	Water Potential (kPa)									
	-220		-710		-1,140		-2,140		-3,550	
	$\bar{x}$ (± S.D.)	Range	$\bar{x}$ (± S.D.)	Range	$\bar{x}$ (± S.D.)	Range	$\bar{x}$ (± S.D.)	Range	$\bar{x}$ (± S.D.)	Range
Buried	+31.8	-	+20.0 (13.6)	+14.1- +32.0	+18.8 (5.2)	+10.2- +25.2	+ 2.9 (1.5)	+ 1.0- + 4.9	-16.4 (6.2)	-10.6- -23.3
Surface	+12.0 (5.3)	+ 5.5- +18.4	+ 6.4 (4.8)	+ 1.1- +13.8	+ 3.0 (1.5)	+ 1.6- + 5.1	- 6.3 (1.9)	- 3.9- - 8.5	-20.9 (5.2)	-12.7- -27.4
Rack	- 9.6 (2.9)	- 6.2- -13.3	-10.4 (2.7)	- 6.4- -13.8	-12.0 (2.5)	- 8.8- -15.3	-14.0 (6.2)	- 7.3- -26.6	-21.2 (6.2)	-14.6- -27.5

There is no significant difference in the weight loss of each of the groups of eggs suspended above the substrate (single factor ANOVA) because of the wide scatter in the data, as indicated by large standard deviations. However, the mean weight loss data (Fig. 6.8) show a trend for eggs above drier substrates to lose more water than those above wetter ones.

There is no significant difference ($t = 0.115$, $p>>0.50$) between the amount of weight lost by eggs on the surface of, and those suspended above, the -3,550 kPa substrate.

Changes in the Mass of Eggs as a Function of Substrate Water Potential

There is a significant positive correlation between the weight change of eggs during incubation and the water potential of the substrate for eggs buried in, and half-buried in the incubation substrates (Figs. 6.10, 6.11) between -220 and -2,140 kPa ($p<<0.005$ in both cases). For eggs suspended on racks the slopes of regressions relating weight to substrate water potential and to the relative humidity of the atmospheres above those substrates (Fig. 6.12) are significantly different from zero ($0.01<p<0.025$ in both cases) but the low r^2 values (Table 6.6) indicate that the fit of the lines is not significant.

TABLE 6.6

Trial	Slope	Y-intercept	r^2	Significance
Buried	0.0155	36.282	0.807	$p<<0.005$
Surface	0.0093	13.540	0.823	$p<<0.005$
Rack	0.0024	- 8.975	0.170	$0.01<p<0.025$
Rack (RH)	3.378	-346.773	0.170	$0.01<p<0.025$

Linear regressions of percent weight change against water potential of the incubation substrate and relative humidity (RH) of the atmosphere above the substrate for eggs on racks. Significance tests refer to comparison of slope to zero using ANOVA.

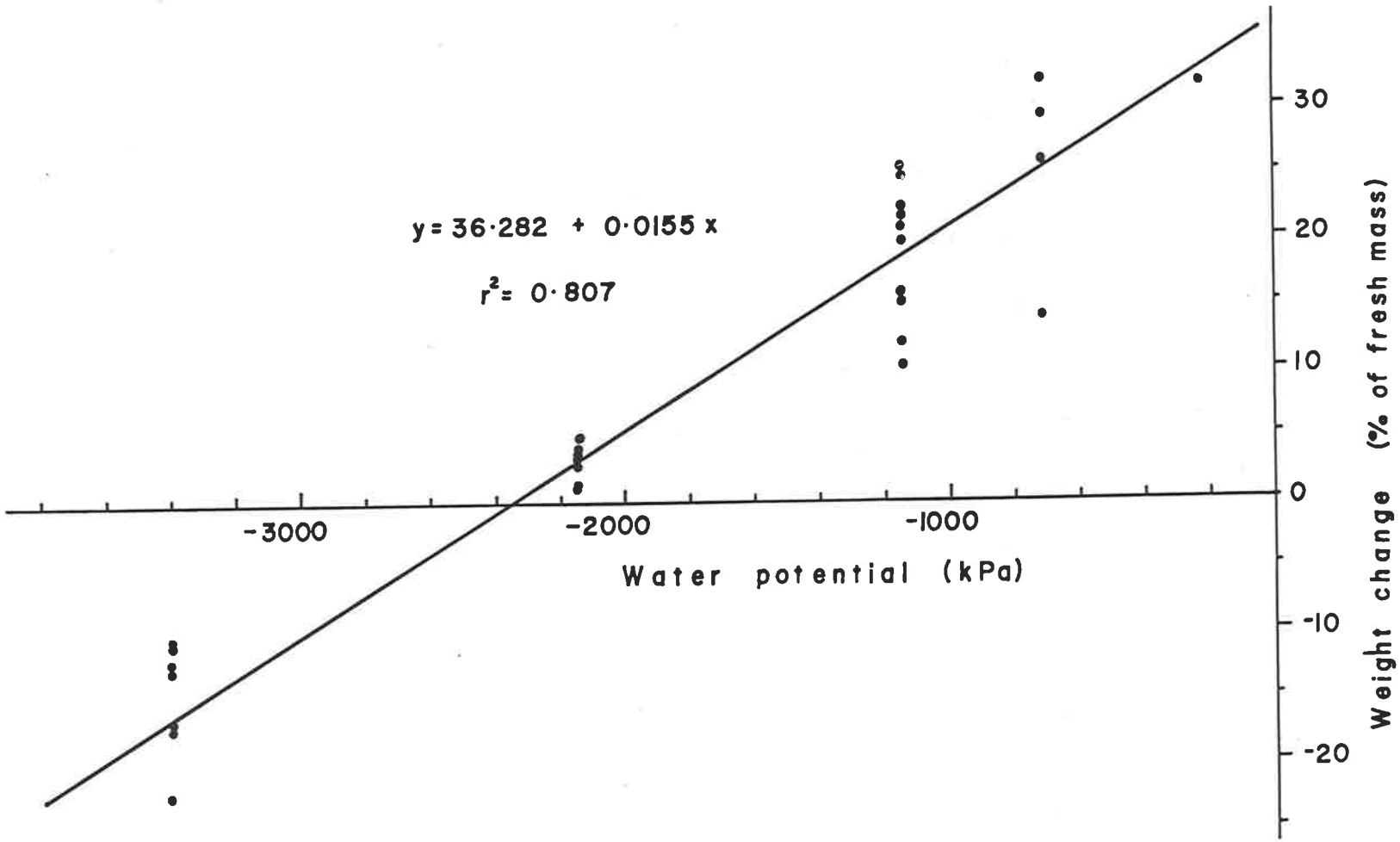


FIGURE 6.10 WEIGHT CHANGE (% OF FRESH WEIGHT) OF EGGS COMPLETELY BURIED IN INCUBATION SUBSTRATE EXPRESSED AS A FUNCTION OF THE WATER POTENTIAL OF THE SUBSTRATE. REGRESSION LINE OF POINTS FROM -220 - -2,140 kPa IS FITTED. POSITIONING OF POINTS AT -3,400 kPa WAS DETERMINED BY INSERTING THE MEAN WEIGHT LOSS INTO THE REGRESSION EQUATION AND SOLVING FOR WATER POTENTIAL (see text for details).

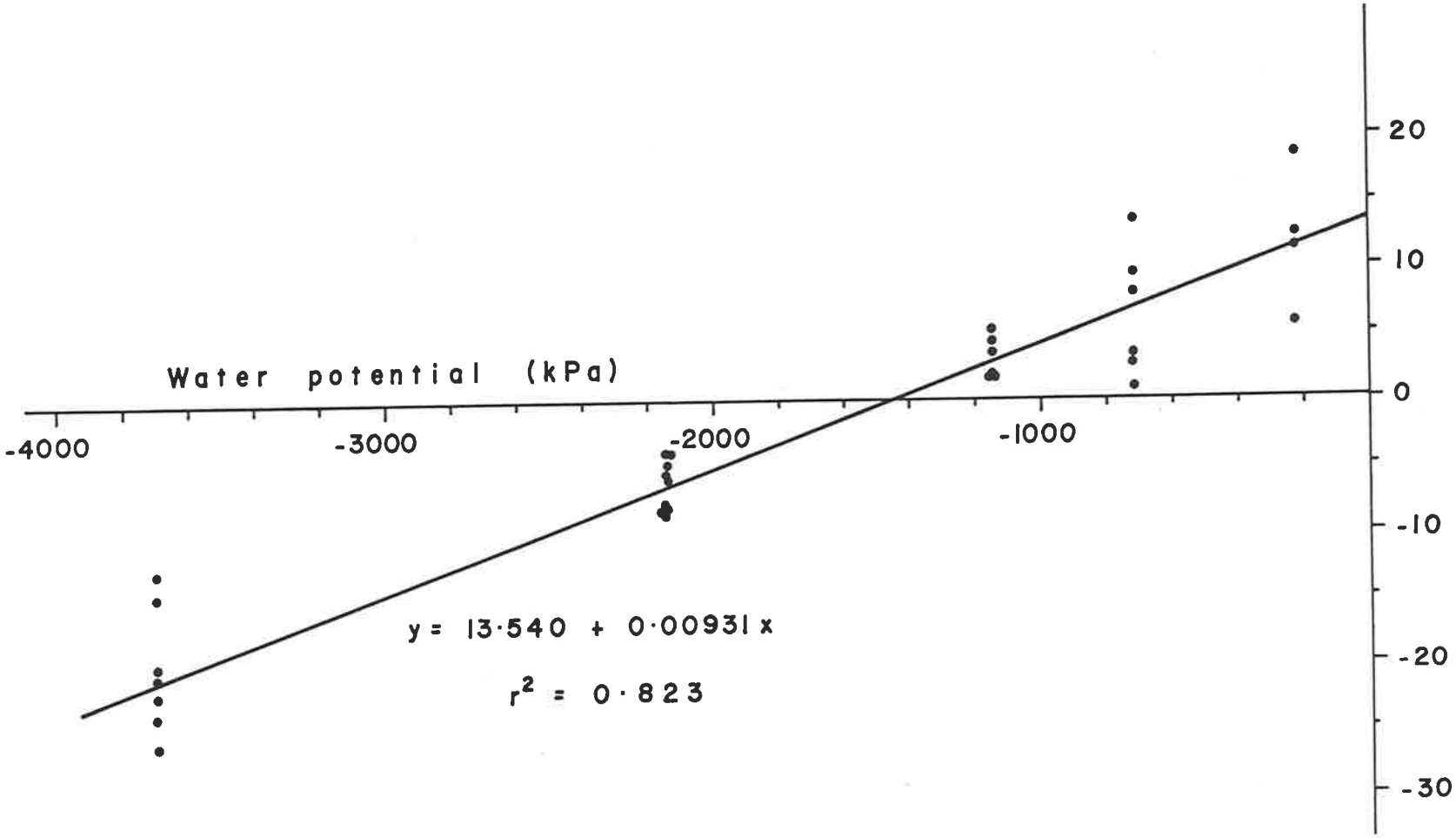


FIGURE 6.11 WEIGHT CHANGE (% OF FRESH WEIGHT) OF EGGS HALF BURIED IN THE INCUBATION SUBSTRATE AS A FUNCTION OF THE WATER POTENTIAL OF THE SUBSTRATE. REGRESSION LINE OF POINTS FROM -220 - -2,140 kPa IS FITTED. POSITION OF POINTS AT -3,700 kPa WAS DETERMINED BY INSERTING THE MEAN WEIGHT LOSS INTO THE REGRESSION EQUATION AND SOLVING FOR WATER POTENTIAL (see text for details).

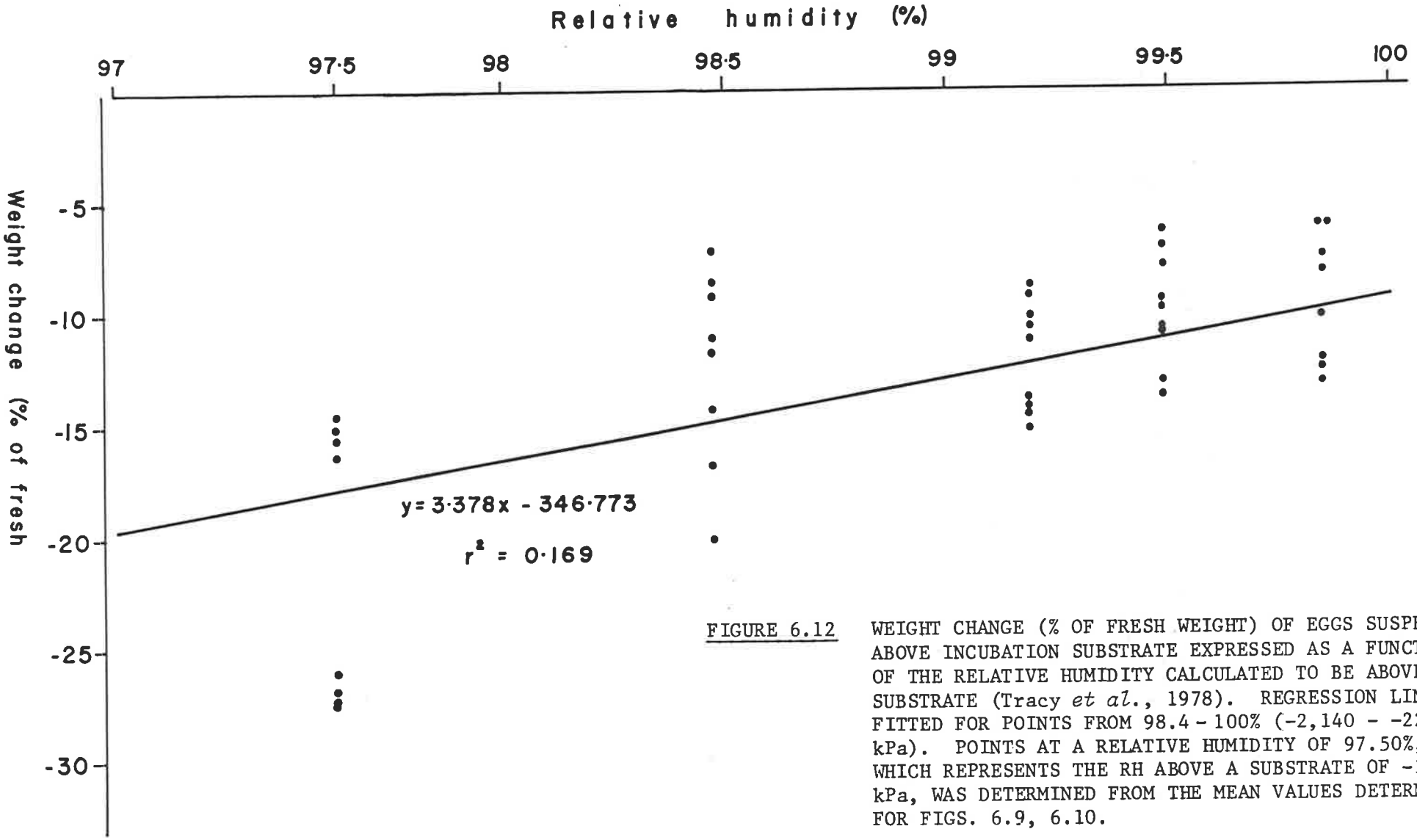


FIGURE 6.12 WEIGHT CHANGE (% OF FRESH WEIGHT) OF EGGS SUSPENDED ABOVE INCUBATION SUBSTRATE EXPRESSED AS A FUNCTION OF THE RELATIVE HUMIDITY CALCULATED TO BE ABOVE THE SUBSTRATE (Tracy *et al.*, 1978). REGRESSION LINE IS FITTED FOR POINTS FROM 98.4 - 100% (-2,140 - -220 kPa). POINTS AT A RELATIVE HUMIDITY OF 97.50%, WHICH REPRESENTS THE RH ABOVE A SUBSTRATE OF -3,550 kPa, WAS DETERMINED FROM THE MEAN VALUES DETERMINED FOR FIGS. 6.9, 6.10.

Water Potential of Egg Contents

The water potential of the egg contents falls gradually between days 1 and 30 (Fig. 6.13). Initially the albumen fraction has a higher water potential than the yolk but by day 8 the potentials are similar, with albumen being slightly lower than yolk. However these trends are represented by only three eggs. Water potentials of mixed fractions indicate that this may be an oversimplification.

6.3.3 Pyramid Experiment

For convenience eggs sitting on other eggs are referred to as "trial eggs" and those sitting on marbles as "control eggs".

Significantly higher relative specific activity was recorded in eggs on the substrate than trial eggs, with no significant difference between trial eggs and control eggs (Table 6.7). The calculated equilibrium activity (~ $10\mu C.1^{-1}$) was double the actual specific activity of the eggs on the substrate and was equal in all three chambers.

TABLE 6.7

	ES	EE	EC
n	25	10	10
$\bar{x}$	5.332	3.831	3.829
S.D.	± 0.860	± 0.667	± 1.060
Significance	***		NS

Relative specific activity ($\mu C \cdot 1^{-1}$) of the water in the albumen of eggs in the pyramid experiment. ES = eggs on the substrate, EE = trial eggs on ES eggs, EC = control eggs on marbles.
*** = $p<<0.001$ ($t_{33} = 4.942$), NS = not significant.

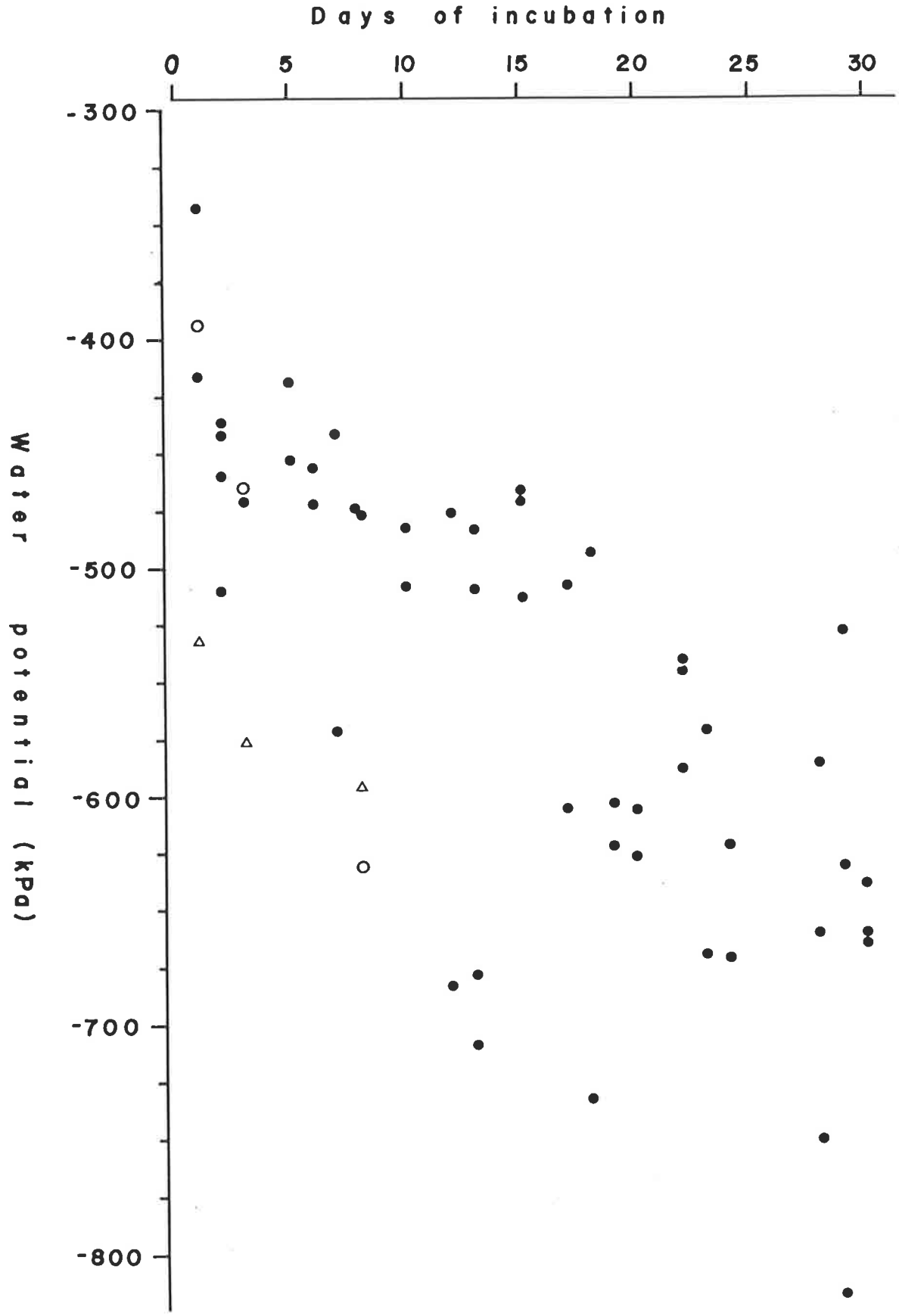


FIGURE 6.13 THE WATER POTENTIAL OF THE YOLK AND ALBUMEN OF 53 EGGS INCUBATED SITTING ON SUBSTRATES OF -1,140 kPa AT 30°C. ○ REPRESENTS ALBUMEN FRACTION, △ REPRESENTS YOLK, ● REPRESENTS MIXED YOLK AND ALBUMEN.

Within four hours of being laid small water droplets form on the surface of the eggs, as if they are sweating. This surface water is gradually lost, presumably by evaporation to the atmosphere. The last place to lose it is the point of contact between eggs where surface water is visible up to 24 hours later.

Fluoroscein

Fluoroscein was detected on the outside and inside of the eggshell of all eggs contacting the substrate. The fluoroscein had a mottled distribution on the inside of the eggshell, indicating that liquid water flow into the egg is not even. No fluoroscein was detected on that part of the shell not contacting the substrate or on any part of the shell of the trial or control eggs.

The albumen from all eggs contacting the substrate fluoresced strongly. In one the fluorescence was at a lower level. Fluoroscein was not detected in the albumen of any of the trial or control eggs.

6.4 DISCUSSION

6.4.1 Field Samples

Searching Behaviour

Probing of soil with the snout prior to nesting and oviposition occurs in some species of chelonians (Carr & Giovannoli, 1957; Carr & Ogren, 1960; Carr & Hirth, 1962; Carr *et al.*, 1966; Moll & Legler, 1971; Bustard, 1972; Burger, 1977) and lizards (e.g. *Agama agama*, Harris, 1964; *Anolis aeneus*, Stamps, 1976). These species all lay soft-shelled eggs. This behaviour may be a testing of the moisture content of the substrate, which may be important for species, such as *Chelydra serpentina* and *Chrysemys picta*, in which incubation on moister substrates results in larger hatchlings (G. Packard *et al.*, 1980, 1981a). Because larger hatchlings have a competitive and selective advantage over smaller ones (Froese & Burghardt, 1974; Tucker *et al.*, 1978; Swingland & Coe, 1979), some gravid female chelonains (and other reptiles laying soft-shelled eggs) may be under selection for their ability to locate nest sites of optimal water potential (M. Packard *et al.*, 1982a).

In *E. macquarii* no selection of nest sites (Fig. 6.3) or soil probing is evident, and nesting occurs in all soil types around Lake Bonney. Many partly dug nest holes were found during the study but they usually appeared to have been abandoned because of a rock or root preventing proper nest construction. The hard-shelled eggs of *E. macquarii*, are tolerant of a wide range of hydric conditions with eggs hatching after net changes in water content ranging from -27.5 to +32.0% of the fresh egg mass. Despite this, the final egg mass (and therefore the hydric condition of the incubation medium) is not related to the size of the hatchlings. Therefore female *E. macquarii* should not experience selection pressure to choose nesting sites according to soil characteristics.

The water content of the substrate adjacent to nests (Fig. 6.4) reflects the different water potential characteristics of the soil used by *E. macquarii* for nesting. One nest is conspicuous in retaining a high percentage of water throughout the incubation period; this nest was in clay rather than the more common sandy loams or sand, and may even have had a lower water potential at the end of the season than the nests in sandy soil. As all the nests hatched successfully, hatching is not impaired by the water potential characteristics of the soil, even at the extremes.

All nests experienced a drop in water potential with incubation time. The female *E. macquarii* usually lay their eggs during or just after rain (Goode, 1965) as do other species (Vestjens, 1969; Burger & Montevecchi, 1975; Plummer, 1976; Cox & Marion, 1978). As the summer progresses, rains become less frequent and temperatures rise causing a gradual drying of soils.

The Nest

The stereotyped method of nest construction in chelonians has some adaptive advantages. The primary importance seems to be the completion of a nest that is sealed to the atmosphere by the nest plug and has an egg chamber that is largely free of soil. By constructing a nest with a narrow entrance the animal is less likely to allow soil to enter the egg chamber when covering the nest than if the entrance is the same diameter as the egg chamber. If there is any minor slipping of sand in the nest chamber of *Chelonia mydas* during its construction, the nest is abandoned and another is constructed (Hendrickson, 1958). If nest construction is unsuccessful on one night a female *C. mydas* will return on successive

nights until a successful nest is completed (Bustard, 1972) which suggests they, and possibly all chelonians, expend considerable effort ensuring the egg chamber remains free of sand. If the eggs of *C. mydas* were packed in sand the partial pressure gradient of O_2 and CO_2 between the centre and perifery of the nest would be 2 - 3 times greater resulting in the eggs at the centre of the nest suffering a greater mortality and increased hatching time (Ackerman, 1980). In addition, an open egg chamber enhances the ability of the hatchlings to dig their way to the surface. Hatchlings emerge from the egg into an open chamber. They make their way to the surface in an open space because the roof of the chamber is collapsed by the mass movement of hatchlings, and trampled underfoot (Hendrickson, 1958; Carr & Hirth, 1961). A turtle hatchling, surrounded by soft sand is able to dig its way out only with great difficulty (Carr & Hirth, 1961).

Cloacal Fluid Release

Many unrelated chelonians release fluid from the cloaca when digging nest chambers (e.g. *Chrysemys picta*, Cunningham, 1923; Stromsen, 1923; Mahmoud, 1968; *Pseudemys scripta*, Cagle, 1950; Moll & Legler, 1971; *Terrapene ornata*, Legler, 1960; *Chelodina longicollis*, Vestjens, 1969; *Chelodina expansa*, Goode, 1965). The release of fluid does not occur during every nesting of species in which it has been observed (Vestjens, 1969). The fluid released by *Emys orbicularis* is pond water (Rollinat, cited by Young, 1950), presumably taken into the cloacal bursae through the cloaca.

Careful examination of completed and uncompleted nests gave no indi-

cation that fluid is released by *E. macquarii* although they possess cloacal bursae. However, none were seen constructing nests in this study.

Release of fluid is a widespread phenomenon and may serve a necessary function. The fluid from the desert tortoise (*Gopherus agassizi*) is a deterrent to mammalian predators (Patterson, 1971) yet Moll and Legler (1971) found that predators could locate nests by the smell of urine. If predator deterrence is a primary function it is puzzling that in some species fluid is released some times but not others. One function of cloacal fluid may be to moisten soil used in the nest plug, to make it cohesive enough not to fall into the spaces between the eggs when the nest is being covered. This would account for the patchy records of cloacal fluid release; some soil types would not require wetting. The preparation by marine turtles of a body pit in the loose surface sand before a nest chamber is constructed in the deeper, moister, more cohesive sand indicates the importance of nesting in cohesive sand in these chelonians. Such an explanation is also consistent with the segregation of wet from dry soil during nest construction and the careful placement of wetted soil into the nest entrance by *Chrysemys marginata* (Stromsen, 1923).

6.4.2 Response of Eggs to Substrates of Different Water Potential

The eggs of *E. macquarii* are tolerant of a wide range of desiccating conditions but excess water causes high mortality. In substrates at field capacity all eggs die early in the incubation period, presumably from drowning. A low hatching success rate occurs in the wettest substrates below field capacity (-110, -220 kPa; Table 6.3). Eggs that survive incubation in these substrates are the ones with the lowest rates of water absorption, presumably due to a lower shell conductance.

My experiments do not indicate the length of exposure to free water necessary to kill an egg or how the sensitivity to flooding conditions is likely to alter with the stage of development of the embryo. Submersion of eggs of *Trionyx muticus* for up to 24 hours in the early stages of incubation does not significantly alter their hatching success. However longer periods of submersion result in a greater number of deaths with submersion for eight days killing all eggs (Plummer, 1976). Later stages of embryonic development are likely to be more susceptible to drowning due to their higher O_2 demand. Drowning is a major cause of egg mortality in other chelonians and crocodilians (Ragotskie, 1959; Roze, 1964; Vestjens, 1969; Joanen, 1969; Chadbreck, 1973; Webb *et al.*, 1977). *Sternotherus minor* selects nesting sites where the possibility of inundation is minimal (Cox & Marion, 1978).

Because all eggs hatch on racks at all water potentials (Table 6.3) it seems unlikely that desiccation is a cause of death to eggs under conditions that exist in an undisturbed nest. Some authors attribute the death of eggs to desiccation (Calge, 1937; Whitaker, 1968; Vestjens, 1969) but other causes of death, such as overheating, can not be ruled out (Ewert, 1979).

Despite the wide range of water potentials experienced by eggs that successfully hatched in this experiment (from buried in -220 kPa to suspended above -3,550 kPa), there was no significant difference in hatchling mass. Changes in mass of eggs incubated in substrates of different water potentials range from an increase of 32% to a loss of 27.5%. This indicates that more than enough water, which includes metabolic water (Ar & Rahn, 1980), for normal incubation is contained within an egg when it is laid. Hence there is no advantage, at least in terms of increasing the size of hatchlings, in the eggs of *E. macquarii* absorbing water during the

incubation period. In *Crocodylus novaeguineae* mass changes ranging from -24.7 to +23.0% due to net water exchange on substrates of different water potential still result in successful hatching (Bustard, 1971b).

Because large hatchling size has a selective advantage (Froese & Burghardt, 1974; Tucker *et al.*, 1978; Swingland & Coe, 1979; G. Packard *et al.*, 1981b) it would be interesting to know whether water flows between eggs in species, such as *Chelydra serpentina* and *Chrysemys picta* which give rise to larger hatchlings if incubated in moister substrates (Tracy *et al.*, 1978; G. Packard *et al.*, 1980, 1981a). If there is no water flow between eggs then there is an advantage in being at the edge of the nest which would result in selection for small clutches so that all eggs could contact soil (G. Packard *et al.*, 1981a). However, other advantages accruing from having large clutches must outweigh the disadvantages of having eggs in the middle of the nest. Although hatchlings emerging from the eggs of *C. picta* incubated in contact with moist substrates are larger than those from drier substrates, there is no difference in the size of hatchlings from eggs suspended above substrates of different water potential (G. Packard *et al.*, 1981a). This implies that there is no difference in the size of hatchling *C. picta* from eggs in the centre of different nests regardless of the water potential of that substrate. In contrast, larger hatchlings emerge from eggs of *Chelydra serpentina* incubated on racks experiencing higher relative humidities (G. Packard *et al.*, 1980).

Eggs of *Chelydra serpentina* incubated in wetter environments take longer to hatch and utilize their yolk reserves more fully than those in drier substrates (G. Packard *et al.*, 1981b). There is no evidence of differential hatching times in different trials in *E. macquarii* and therefore no difference in the utilization of yolk reserves is implied.

Similarity in the wet or dry masses of hatchlings of *E. macquarii* between trials strongly supports this suggestion. The incubation water potential probably does not affect the incubation time of the eggs of the lizard *Dipsosaurus dorsalis* either (Muth, 1980).

There are wide standard deviations in the weight changes in all groups of *E. macquarii* eggs (Table 6.5, Figs. 6.6 - 6.8). These reflect the high degree of variation in the eggshell conductances (Chapter 5) which result partly from variations in shell thickness (Chapter 3). Significant differences in the rate of water storage ($\dot{m}_{st}$) also occur between eggs of *Chrysemys picta* incubated in substrates of equal water potential (Tracy *et al.*, 1978).

The air in all experimental trials was close to saturated with water vapour (Table 6.3). The small differences in relative humidity made very little difference to the amount of water lost by suspended eggs. A trend for eggs over drier substrates to lose more water than those over moister substrates is indicated by the regressions relating weight loss to soil water potential and to relative humidity, which have slopes significantly different from zero (Table 6.6). However the low correlation coefficient indicates a wide variation in the results and shows that this trend is probably not biologically significant.

Eggs in natural nests probably experience water vapour tensions of the order used in this experiment (Table 6.3), i.e. near to saturation (Hillel, 1973). The air in alligator nests is almost saturated with water vapour and is relatively constant throughout the incubation (Joanen, 1969). Probably the only water requirement for the successful hatching of hard-shelled reptilian eggs is a high relative humidity (>95%) (G. Packard *et al.*, 1981c). Any further water available may be used to buffer any

future falls in available water (Clark, 1953a; Fitch & Fitch, 1967; Badham, 1971; Bustard, 1971b). Successful hatching in *Trionyx spiniferus* is thought to depend only on there being about 100% relative humidity in the nest chamber (G. Packard *et al.*, 1981c). Transpirational water loss in the hard-shelled eggs of *T. spiniferus* in natural nests probably constitutes a relatively small and constant fraction of the total water reserve present in the albumen and is largely independent of the water content of the substrate (G. Packard *et al.*, 1979a). This is the situation found in most birds where a constant fraction of the water store in the egg is lost prior to hatching (Rahn & Ar, 1974) and the relative humidity experienced by the eggs is probably also maintained at a fairly constant level by the hen sitting on the eggs (Rahn *et al.*, 1976).

It is interesting that there was no significant difference between amount of water lost by eggs suspended above and those sitting on a substrate of -3,550 kPa. Both groups have similar ranges of weight loss with a mean of 21% (Table 6.5). An average weight loss of 21% maybe quite normal for eggs of *E. macquarii*. This value is close to the 14 - 18% weight loss experienced during the incubation of avian eggs (Rahn & Ar, 1974; Rahn *et al.*, 1976; G. Packard *et al.*, 1977b). Eggs of *Chrysemys picta* suspended on racks above -130 kPa lose 7% and above -610 kPa 15% of the initial egg mass which is also comparable to losses in avian eggs (G. Packard *et al.*, 1981a).

Although the eggs of *E. macquarii* are able to sustain losses of up to 27% of their weight (Table 6.5) they probably seldom experience losses of this magnitude in the field. Such large losses occur only in eggs incubated in the driest substrates for the entire incubation period. In the field, water potentials are relatively high early in the incubation period, probably of the order of -700 kPa, and fall to values of the order of

-3,550 kPa during incubation (Fig. 6.4). Hence eggs in the field are not subjected to constant low water potentials and are therefore unlikely to lose as much as 27% of their weight before hatching. Predictions of the amount of water that eggs in the field are likely to lose during incubation are impossible because of the continual changes to the soil water potential with rain and vapour pressure with temperature changes (Hillel, 1973).

G_{H_2O} calculated for eggs of *E. macquarii* incubated on racks above -3,550 kPa (i.e. 97.5% R.H.) is about three times that predicted from G_{H_2O} values for eggs over silica gel (Chapter 5). G_{H_2O} measured by the rate of water loss from eggs in conditions of known and constant relative humidity (R.H.) is much higher at high R.H. than at low R.H. (Seymour, unpublished data). Measurements of G_{H_2O} are usually made by placing eggs in conditions of zero humidity and constant temperature, thus creating a large vapour pressure gradient between the egg contents and the dry atmosphere; 23.7 torr at 25°C. However, in conditions of high R.H. (e.g. eggs suspended above substrates of -3,550 kPa, 97.5% R.H. at 30°C) the vapour pressure difference is small (0.8 torr in this case) and the rate of water loss is much smaller than in desiccating conditions. The slow rate of water loss may allow water to move, by capillarity, towards the surface of the shell through the pores. Thus the evaporative surface, instead of being at the surface of the inner shell membrane, as usually assumed, is much closer to the surface of the shell, the distance through which diffusion occurs is smaller, and the apparent G_{H_2O} is higher. The higher G_{H_2O} in the eggs of *E. macquarii* at 97.5% R.H. than at 0% R.H., and data for eggs of the brush turkey, *Alectura lathami* (Seymour, unpublished data), support this hypothesis.

Metabolic heat may also contribute to higher G_{H_2O} in eggs suspended

on racks because they all survive to hatching, whereas those over silica gel do not. However, this heat production is probably insufficient to account for the whole difference in G_{H_2O} measured at 0% R.H. and 97.5% R.H.

The initial net loss of mass experienced by all eggs in the experiments is assumed to be due to water loss from the pores in the shell. It takes about a third of the incubation period for the eggshell to dry out, i.e. for the white patch to reach its maximum development (see Fig. 5.13). Further losses are assumed to be water from the albumen fraction of the egg. Accelerated rates of water absorption begin between 30 and 85% of the way through the incubation period (Figs. 6.6, 6.7). As the egg absorbs water and the shell cracks, the conductance of the shell and membranes must increase and may account for the accelerated rates of weight gain. Rates of water loss do not change throughout the incubation of eggs experiencing a net loss prior to hatching (Figs. 6.7, 6.8).

In *E. macquarii* all eggs lose water for at least one third of the incubation which is similar to *C. picta*, where all eggs, except those on -130 kPa, lose water for at least the first four days (G. Packard *et al.*, 1981a). In *E. macquarii*, even eggs incubated buried in a substrate of 15.8% water (= -110 kPa) lose water for at least seven days and do not make up their fresh mass before 18 days at 25°C. The water potential of the albumen on day 1 is -395 kPa (Fig. 6.13) and the embryonic respiration is too low to produce any significant heat (Chapter 4). Consequently eggs in substrates of water potentials greater than -395 kPa (e.g. -110 kPa) should gain water. How water is lost against this gradient is not understood. However, the small droplets of water that appear on the surface of eggs of *E. macquarii* after laying, may be water forced from the pores by hydrostatic pressure in the eggs that is neutralised by pressure in the

body cavity of the female while the eggs are in the oviducts.

In experiments with the eggs of *Trionyx spiniferus* incubated on and above substrates of a variety of water potentials (-50 - -900 kPa) all eggs lose water and no eggs crack (G. Packard *et al.*, 1979a, 1981c). However, cracks develop in naturally incubating eggs of *Trionyx muticus* (Plummer, 1976), alligators (McIlhenny, 1935; Ferguson, 1982) and crocodiles (Webb *et al.*, 1977). I also noted them in eggs of *E. macquarii* and *Chelodian longicollis* incubated in moist substrates in the laboratory as did Iverson (1977) in the eggs of *Kinosternon subrubrum*. Hence the suggestion of G. Packard *et al.* (1981c) that the uptake of sufficient water to cause cracking in hard-shelled eggs in nature is not a usual occurrence should be viewed with caution.

The artificial incubation experiments on eggs of *E. macquarii*, suggest that they behave in a manner intermediate between the hard-shelled eggs of *Trionyx spiniferus* (G. Packard *et al.*, 1979a, 1981c) and the hard, expansible-shelled eggs of *Emydoidea blandingii* (G. Packard *et al.*, 1982) as originally suspected. In summary the similarities shared with *T. spiniferus* but not *E. blandingii* are:

(1) there is no difference in hatchling mass from eggs incubated in conditions differing in water potential;
(2) mortality is spread evenly among trials (except in very wet conditions); and
(3) the rate of change of water storage ($\dot{m}_{st}$) increases throughout the incubation period.

The similarity shared by *E. macquarii* and *Emydoidea blandingii* but not *T. spiniferus* is:

eggs gain water at high water potentials and in so doing crack and swell to accomodate the extra fluid.

6.4.3 Water Storage

The total amount of water absorbed by eggs of *E. macquarii* buried in, and sitting on, substrates ranging in water potential from -220 to -2,140 kPa is a linear function of substrate water potential, similar to the eggs of *Chrysemys picta* sitting on substrates of different water potential (Tracy *et al.*, 1978).

The increasing water storage in eggs of *C. picta* was found to be a curvilinear (quadratic) function of incubation time (Tracy *et al.*, 1978) but further work has indicated that this was in error (G. Packard *et al.*, 1981a). In the eggs of *E. macquarii* buried in, and sitting on, the substrate the relationship is not linear and does not approach an asymptote. Increasing rates of water absorption by buried eggs sitting on the wettest substrates may be due to the decreasing hydraulic resistance of the shell as it increases in area and the shell cracks with the uptake of more water. There becomes a point where the rate of uptake must approach zero as the water potential of the egg contents approach the water potential of the substrate. However, embryonic death occurs before any reduction in the rate of water storage is observed in eggs buried at -220 kPa.

6.4.4 Test of Water Storage Equation

Tracy *et al.* (1978) assume that water is lost only from that part of the egg exposed to the atmosphere and gained by that part contacting the substrate. They measured water loss in eggs totally exposed to air (m_a) and weight changes of eggs with 50% of their surface contacting the substrate and 50% exposed to the air (m_{st}). By dividing m_a by two and adding this value to m_{st} they calculated the total water absorbed (m_s) using equation 6.1. If this method is correct, calculated values of m_s should be the same as the weight changes measured in eggs completely buried in

the incubation substrate. There is close agreement between the two sets of values, i.e. m_s (calculated) and m_s (measured), for the eggs of *E. macquarii* (Table 6.8), which supports the idea that water is lost from that part of the egg exposed to air and is absorbed through contact with the substrate.

TABLE 6.8

Water Potential (kPa)	m_s (percent of fresh weight)	
	Calculated	Measured
- 220	33.622	31.820
- 710	23.174	25.274
-1,140	18.052	18.808
-2,140	1.396	2.963
-3,550	-20.536	-14.797

Total water absorbed (m_s) (percent of fresh egg weight) by eggs of *E. macquarii* at five water potentials. The two values are: (1) calculated m_s of buried eggs based on measurements of m_a and m_{st} of eggs suspended on racks and with half the surface area contacting the substrate using the theory of Tracy *et al.* (1978), and (2) values of m_s measured in buried eggs (m_a assumed to be zero).

6.4.5 Water Potential of Egg Contents

At laying the water potential of the albumen is -395 kPa and of the yolk is -531.2 kPa in *E. macquarii*. These values are similar to those for *Chrysemys picta* which result in water movement from the albumen to the yolk by osmosis (G. Packard *et al.*, 1981a). In *E. macquarii* the water potential of mixed egg contents, excluding the embryo, falls consistently from day one to day 30. There is a range of water potentials in eggs of any one age (Fig. 6.13). In *C. picta* the water potential of the contents

of the vitelline sac rises to -384 kPa by day 11 and then falls again to -501 kPa by day 22.

An initial aim of this study was to incubate eggs buried at different water potentials to establish the water potential of the eggs by monitoring water movements. The results (from Fig. 6.6) indicate that the water potential of the egg is slightly less than -2,140 kPa. This estimate is far lower than the measured water potential of the egg contents. The theoretical calculations of Tracy *et al.* (1978) predict a water potential for the contents of fresh eggs of *Chrysemys picta* of -706.6 kPa which is in the range of values found in the blood of *C. picta* (Dentzler & Schmidt-Neilsen, 1966). This is much lower than the lowest value measured by G. Packard *et al.* (1981a) i.e. -557 ± 35 kPa in yolk on day 1 of incubation. Therefore measurements of water movement in eggs in substrates of known water potential cannot be used as a reliable indication of the water potential of egg contents. Eggs of *E. macquarii* buried in substrates of -2,140 kPa and higher (i.e. wetter) experience a net gain of water over the incubation (Fig. 6.6). However, the minimum water potential of the egg contents is -818 kPa at day 29 of the incubation on a substrate of -1,140 kPa (Fig. 6.13). Therefore the eggs of *E. macquarii* absorb water against a potential gradient of at least 320 kPa, and those of *C. picta* against a potential gradient of 150 kPa (G. Packard *et al.*, 1981a). The mechanism of water uptake against such a large gradient is unknown. The gain of water by eggs of *E. macquarii* against a gradient continues to the end of the incubation (Figs. 6.6, 6.7) despite the increasing metabolic heat production whereas metabolic heat reverses the rate of water gain against the gradient in the eggs of *C. picta* (G. Packard *et al.*, 1981a).

Water uptake is rapid during the first three days in the soft-shelled eggs of the lizard *Amphibolurus barbatus* and then continues at a reduced

rate for the rest of the incubation period (Badham, 1971). The initial high rate of water uptake is due to the osmotic concentration of the yolk. With the placement of a layer of albumen between the yolk and the shell the rate of water uptake decreases. The yolk passes unhydrated protein molecules through the vitelline membrane, which is semi-permeable in lizard eggs, to the albumen as it absorbs water, thereby maintaining its concentration. The water content of the yolk remains constant throughout incubation. In this way the absorbed water acts as a buffer against variations in ambient water (Badham, 1971). This was also suggested for the eggs of lizards and snakes generally (Fitch & Fitch, 1967) and for *Crocodylus novaeguineae* (Bustard, 1971b). The addition of hydrophylic molecules to albumen strongly binds the water molecules it contains and, because of the high affinity of the colloidal albumen for water, ensures the rapid uptake of water when environmental conditions are favourable for absorption.

The eggs of the lizard *Dipsosaurus dorsalis* generally gain water for the first two thirds of the incubation and then lose some of it before hatching (Muth, 1981a). The water potential of infertile eggs is -250 kPa on day 1 and the water potential and hydraulic conductance fall throughout the incubation period. In agreement with M. Packard *et al.* (1980) for the lizard *Callisaurus draconoides*, Muth suggested that differences in the quantity of water absorbed are due to:

(1) the amount of surface area contacting the substrate; or

(2) the water potential of the egg contents; or

(3) metabolic and transpirational rates; or

(4) shell structure and hydraulic conductance; or

(5) a combination of these.

He was unable to choose between the alternatives. However, he found that

the explanation of G. Packard *et al.* (1977a) and Tracy *et al.* (1978) that net water loss in the last part of incubation is due to increased metabolic heat is not valid. Instead he suggests that, as the metabolic heat of the egg increases, the embryo concentrates solutes in the albumen and yolk sac which is ventrally located and in contact with the chorion. The temperature and vapour pressure difference between the dorsal surface of the egg and the air drives water from the egg. The osmotic potential of the ventral compartments may be low enough to compensate for the temperature increase resulting in water being absorbed through the ventral surface. This mechanism allows simultaneous transpiration and absorption of water while maintaining a net water potential below ambient water potential.

This explanation may be correct in circumstances where the water potential of the ventral compartments of the egg are lower than that of the incubation medium. However, it does not explain the movement of water against the observed concentration gradient between egg contents and incubation medium observed in chelonian eggs. In addition, Muth based part of his argument on the conclusion of Tracy *et al.* (1978), that the water potential of the egg comes to equilibrium with that of the incubation substrate in *C. picta*, which was shown to be incorrect by G. Packard *et al.* (1981a). However, G. Packard *et al* (1981a) were unable to describe the mechanisms involved in the pattern of water transport in the eggs of *C. picta* because of lack of information on surface temperatures of eggs, water potentials of liquids adjacent to the shell membrane and changes in the shell with swelling as water is absorbed.

In *C. picta* the rate of weight gain does not increase until hatching (G. Packard *et al.*, 1981a) as it does in *E. macquarii* incubated in or on wet substrates (Figs. 6.6, 6.7). All eggs of *C. picta* lose weight during

the last week of incubation even in eggs sitting on substrates of -130 kPa. Water vapour exchanges of some reptilian eggs are probably influenced by the water potential of the egg contents early in the incubation period and also by the metabolic heat production in later stages (G. Packard *et al.*, 1981a; Muth, 1981a). High metabolic heat production may explain the loss of water from eggs of *C. picta* just prior to hatching. However, no such loss occurs in the eggs of *E. macquarii*.

6.4.6 Abnormal Hatchlings

Dehydrating conditions during incubation produce abnormalities in the chelonians *Chelydra serpentina* and *Chrysemys picta* (Lynn & Ullrich, 1950; Tracy *et al.*, 1978) and the lizard *Dipsosaurus dorsalis* (Muth, 1980). Careful examination of all hatchling *E. macquarii* incubated in artificial conditions revealed no abnormalities of the kinds described by Lynn and Ullrich (1950). The only abnormalities found were in eggs from one clutch. This clutch had been split up so that eggs were incubated in, on and above substrates of -220, -2,140 and -3,550 kPa. Hatchlings and advanced embryos were all deformed regardless of the water conditions experienced during incubation which suggests that the cause was genetic and not environmental. All hatchlings from this clutch died. Although the species studied by Lynn and Ullrich lay soft-shelled eggs they suggested that the same should occur in species with hard-shelled eggs. My results do not support this suggestion.

6.4.7 Pyramid Experiment

The tritium and fluorescein experiments show that, within the first 48 hours of incubation, liquid water flows from the incubation substrate into eggs contacting the substrate but no detectable flow occurs between

eggs, despite the presence of water bridges. The control eggs, and those sitting on eggs, exchange water with the atmosphere only (i.e. in the gaseous phase), as indicated by their relative specific activity, whereas the higher specific activity of the eggs contacting the substrate indicates that those eggs also exchange water with the substrate.

Fluorescence detected in the eggs contacting the substrate indicates that liquid water flows from the substrate into the egg. The data for eggs on substrates of -710 kPa (Fig. 6.7) indicate that these eggs experience a net loss of water for the first half of the incubation period. Eggs in tritium trials lost a mean of 0.29% of their mass in the first 48 hours. Liquid water exchange with, or absorption from, the substrate is obviously not sufficient to replace water lost from the egg. This simultaneous water gain and loss was postulated by G. Packard *et al.* (1977a) and Tracy *et al.* (1978) who suggest the likely direction for the movement of water is into the egg from the substrate and out of the egg to the nest atmosphere (equation 6.1).

6.4.8 General Discussion

There has been confusion regarding the nature of the hard-shelled eggs of reptiles (e.g. Young, 1950), i.e. are they cleidoic in the sense of Needham (1931)? Needham (1931, 1942) thought that the eggs of the Crocodilia were cleidoic and those of the Chelonia non-cleidoic. On the basis of this suggestion Young (1950) measured the rate of water loss from eggs of the Greek tortoise, *Testudo graeca*, compared it to that of chicken eggs and concluded that tortoise eggs were cleidoic in the sense of Needham. Eggs of the American alligator, *Alligator mississippiensis*, swell and in so doing crack to allow expansion, presumably as a result of water uptake (McIlhenny, 1935; Joanen, 1969; Ferguson, 1982). The eggs

of *Crocodylus novaeguineae* also gain water during incubation on a moist substrate (Bustard, 1971b). Hence crocodilian eggs could not be truely cleidoic.

The hard-shelled eggs of *Trionyx spiniferus* are effectively and functionally cleidoic under normal conditions of incubation and this is probably true for all hard-shelled chelonian eggs (G. Packard *et al.*, 1979a, 1981c). In contrast the soft-shelled eggs of *Chrysemys picta* and *Chelydra serpentina* are non-cleidoic (Tracy *et al.*, 1978; G. Packard *et al.*, 1980, 1981a). The responses of the eggs of *Emydoidea blandingii* to the hydric conditions of incubation are intermediate between those of hard-shelled and soft-shelled species (G. Packard *et al.*, 1982). Hence there is a range in the capacities of eggs of different species of chelonians to exchange liquid water with the environment.

It has long been known that to artificially incubate reptilian eggs a "moist" incubation substrate must be provided (Lynn & von Brand, 1945; Clark, 1946, 1953a). The literature indicates that there is a tolerance to a wide range of moisture conditions resulting in successful hatching within a species (e.g. Bustard, 1971a,b; Ernst, 1971a) and that eggs incubated in moist substrates often give rise to larger hatchlings than those in drier substrates (Cunningham & Huene, 1938; Tracy *et al.*, 1978; M. Packard *et al.*, 1980; G. Packard *et al.*, 1980, 1981a,b, 1982). These observations have led to many laboratory experiments on the water relations of reptilian eggs and the postulation of the possible evolutionary significance of the results (e.g. Clark, 1953a; G. Packard *et al.*, 1981a). Until recently most of these studies were qualitative. The water potentials of the incubation substrates were not reported and so the relationship to natural systems cannot now be determined (G. Packard *et al.*, 1977a). However, future work can be compared to several recent quantita-

tive studies on the water relations of chelonian and lizard eggs.

Although the definition of the term "cleidoic" makes a clear cut distinction between groups of eggs, the classification has proved more complex than indicated. The degree of exchange of liquids with the environment clearly varies with species but the term "non-cleidoic" takes no account of this. In addition the grouping of hard-shelled and cleidoic eggs as one and the same is incorrect. Both classifications (cleidoic / non-cleidoic and hard-shelled / soft-shelled) represent the extremes of a range of egg forms with intermediates at all levels between the extremes. These arbitrary classifications are useful generalisations for comparative discussions of egg types but they do not represent a complete description of the complete range of egg types. Most avian eggs can be regarded as cleidoic (one end of the spectrum) and all soft-shelled eggs as non-cleidoic. However, I define eggs, such as those of *E. macquarii*, that take up liquid if it is available but if it is not there is no influence on the resultant hatchling, as facultative cleidoic eggs. These are non-cleidoic eggs in which the hatchling is the same regardless of whether the incubation was cleidoic (e.g. if the egg is in the centre of the nest) or non-cleidoic (e.g. at the edge of the nest).

7

EFFECTS OF TEMPERATURE ON EGGS AND EMBRYOS

7.1 INTRODUCTION

The rate of development of reptilian eggs is dependent on temperature. As such, the temperatures experienced by eggs in the field determine the time of the incubation and therefore the length of exposure to other environmental hazards (e.g. Goode & Russell, 1968; Yntema, 1968; Muth, 1980; Miller & Limpus, 1981). There are few records of the temperatures experienced by eggs in the field (Burger, 1976); most determinations of the effect of temperature on incubation time are conducted at constant temperature or "room temperature" in the laboratory. Knowledge of natural temperature regimes have become particularly important since the discovery that the temperature of incubation determines the sex of hatchlings in some reptiles (Bull, 1980).

Sex is genotypically determined in most tetrapod vertebrates (Bull & Vogt, 1979). Heteromorphic sex chromosomes occur in some lizards and snakes (Kobel, 1962; Pennock *et al.*, 1969) but not in crocodilians (Ohno, 1967; Cohen & Gans, 1970; White, 1977, Bull, 1980). Three chelonians have heteromorphic sex chromosomes (Bull *et al.*, 1974; Sites *et al.*, 1979; Carr & Bickham, 1981), but none of the Chelidae have them (Bull & Legler, 1980). Studies of the H-Y antigen, which can be used to determine the heteromorphic sex in species in which the sex chromosomes are not morphologically distinct, show that most chelonians have a ZZ/ZW chromosomal system, and some have an XX/XY system (Zaborski *et al.*, 1979; Engel *et al.*, 1981).

In species with Temperature-dependent Sex Determination (TSD), the temperature at which incubation would result in a sex ratio of unity has been termed the "critical temperature" (Pieau, 1973), "pivotal temperature" (Mrosovsky & Yntema, 1980) and "threshold temperature" (Bull, 1980; Bull *et al.*, 1982). There is a narrow temperature zone for chelonians in

which both sexes are produced, with temperatures greater than the threshold producing more females and temperatures below the threshold producing more males. Outside the threshold zone hatchlings of one sex or the other are produced. In *Chelydra serpentina* and *Sternotherus odoratus* there is a second, lower threshold (<25°C) below which females are produced (Yntema, 1976; Vogt *et al.*, 1982). In contrast to chelonians, alligators and lizards produce males above the threshold temperature and females below it (Bull, 1980).

When incubated at constant temperature in the laboratory the threshold zone is very narrow. Examples are *Emys orbicularis*, 28-29°C and *Testudo graeca*, 30-31°C (Pieau, 1978); *Caretta caretta*, 28-30°C (Yntema & Mrosovsky, 1979, 1980), *Pseudemys scripta*, 28-30°C (Bull *et al.*, 1982) and *Chelydra serpentina*, 28-30°C and 20-22°C (Yntema, 1976). The threshold zones appear to be species-specific (Dimond, 1979).

TSD in reptiles was first recognised in the dragon lizard, *Agama agama* (Charnier, 1966), and subsequently in the chelonians *Emys orbicularis* and *Testudo graeca* (Pieau, 1971, 1972, 1973, 1978) and *Chelydra serpentina* (Yntema, 1978). The marine turtles *Caretta caretta* and *Chelonia mydas* also have TSD (Yntema & Mrosovsky, 1979, 1980; Miller & Limpus, 1981; Morreale *et al.*, 1982) although other factors also may influence sex determination in *C. mydas* (Wood & Wood, 1982). TSD has been shown in 15 out of 16 species of chelonians in six Cryptodiran families (Pieau, 1982). Sex is determined independently of incubation temperature in the lizards *Lacerta viridis* (Raynaud & Pieau, 1972) and *Dipsosaurus dorsalis* (Muth & Bull, 1981) and the turtle *Trionyx spiniferus* (Bull & Vogt, 1979).

The relationship between exposure to temperatures on either side of

the threshold during incubation and the resultant sex ratio of hatchlings is complex (Bull & Vogt, 1979). In the field, shaded nests of *Graptemys* and *Chrysemys* give rise to all males, those in the sun produce all females, and those in partial shade result in both sexes (Bull & Vogt, 1979; Vogt & Bull, 1982a). Nest temperatures in the field also modify the sex ratio in *Emys orbicularis* (Pieau, 1982) and *Chelonia mydas* (Morreale *et al.*, 1982).

The temperature-sensitive stages of development occur during the middle third of the incubation period covering five embryonic stages during organogenesis in *Chelydra serpentina* (Yntema, 1979), *Emys orbicularis* (Pieau & Dorizzi, 1981), *Graptemys ouachitensis* and *Chrysemys picta* (Bull & Vogt, 1981). The sex of hatchling *C. serpentina* does not change within the first three months of life (Yntema, 1976).

H-Y antigens are a cell surface component thought to have a primary role in gonadal sex determination in vertebrates (Harvey & Slatkin, 1982). Studies of H-Y antigens in *Emys orbicularis*, a species with TSD, indicate that all individuals have either a male or a female genotype but that if eggs are incubated at temperatures below the threshold zone, for example, the genotypic females develop as phenotypic males. These individuals have $H\text{-}Y^{+}$ gonadal cells and $H\text{-}Y^{-}$ blood (Zaborski *et al.*, 1982). The converse is true for hatchlings incubated at high temperatures.

There are many reports of biased sex ratios in natural populations of reptiles but such differences have been attributed to non-genetic factors such as differential mortality (Risley, 1932; Forbes, 1964). However, the results of some studies on large numbers of embryos and hatchlings cannot be explained this way (Gibbons, 1970a). For example, female-biased sex ratios were recorded in large numbers of *Malaclemys*

centrata hatched in pens in North Carolina (Hildebrand, 1929, 1932) and in the laboratory (Risley, 1938, 1941a). More females than males have been reported in natural populations of *Sternotherus odoratus* (Risley, 1932, 1933; Cagle, 1942), *Chrysemys picta* (Cagle, 1942; Sexton, 1959), *Pseudemys scripta* (Cagle, 1942, 1950), *Sternotherus depressus* and *S. carinatus* (Tinkle, 1958), *Terrapene ornata* (Legler, 1960), *Geochelone gigantea* (Swingland & Lessells, 1979) and *Emydura macquarii* (Thompson, 1983). TSD may account for some of the variations from equal sex ratio in chelonian populations but very little is known about the sex ratio of adult populations of any species, largely due to the difficulties associated with sampling. However equal sex ratios have been reported in *Sternotherus odoratus* (Tinkle, 1961), *Geochelone gigantea* (Swingland & Coe, 1979) and *Trionyx* (Vogt & Bull, 1982a).

Although complete sex reversal is thought not to be experimentally possible in reptiles (White, 1977), it has not been excluded as an explanation for unusual sex ratios (Forbes, 1964). Incomplete sex reversal can be induced in embryonic *Chrysemys marginata* injected with testosterone proprionate (Risley, 1937, 1940) but not in *Malaclemys centrata* (Risley, 1941a). True hermaphroditic turtles from the field have been reported (Risely, 1941b; Hansen, 1943).

The eggs of *E. macquarii* were incubated at a variety of temperatures and the sex of the hatchlings determined. *E. macquarii* is the first pleurodiran species to be studied in this way. The temperatures at which the eggs were incubated covered all reported threshold zones. These experiments also gave information relating the incubation time to temperature in *E. macquarii* for comparison with other species.

The temperatures of eggs of *E. macquarii* in the field were measured

to determine seasonal and diurnal variations. One reason for this was to establish the maximum temperature of naturally incubating eggs so the maximum rate of respiration could be estimated and used to describe the dynamics of gas exchange between embryo and atmosphere (Chapters 4 and 5). Clutch temperature is known to increase in the nests of marine turtles (e.g. Hendrickson, 1958; Bustard & Greenham, 1968) and *Malaclemys terrapin* (Burger, 1976) due to internal metabolic heating. Accordingly, metabolic heating also was examined in natural nests of *E. macquarii*.

7.2 MATERIALS AND METHODS

7.2.1 Laboratory Experiments

Eggs of *E. macquarii*, collected at Lake Bonney, were divided into batches of eight with one egg from each clutch included in each batch to control for the influence of variation between nests. Three batches were placed in constant temperature cabinets at 25 (± 2)°C, 26 (± 1)°C, 28 (± 0.5)°C, 30 (± 1)°C, and 32 (± 0.5)°C. The eggs were buried in substrates with water potentials of -710, -2,140 and -3,550 kPa (Chapter 6).

Hatchlings were killed with an intraperitoneal injection of Bouin's fluid. The plastron was then cut from its anterior edge to the yolk sac to facilitate fixation and the specimen stored in Bouin's fluid. Sex was determined by dissection under a binocular microscope, according to the criteria of Risley (1933) and Yntema (1976). Wax histological sections of twelve gonads were made and stained with haematoxylin and eosin to confirm gross observations.

The experiment at 30°C gave an anomalous result. To see if the result was consistent sex was determined in 111 additional hatchlings incubated at 30 (± 1)°C.

Twelve eggs induced from one female were divided into two batches of six and incubated at 20°C in sand of -710 and -2,140 kPa. After 91 days of incubation three eggs from each trial were transferred to 30°C (other species develop beyond the sex determining embryonic stages at 20°C but die before hatching unless transferred to higher temperatures (Yntema, 1978; Pieau, 1982)). The remaining six eggs were opened on days 87, 129 (two eggs), 165, 200 and 229 to determine stage of development.

7.2.2 Field Nest Temperatures

During 1978-9, temperatures of three naturally constructed nests (one at Lake Bonney and two at the tortoise farm nearby) were measured by pushing a mercury thermometer into the soil to a depth equivalent to the middle of the egg mass (c. 10 cm) not more than 30 cm from the nest.

Again in 1979-80, six nests (three each at Lake Bonney and the tortoise farm) were partly excavated within 24 hours of oviposition to allow the insertion of copper-constantan thermocouples at three locations in the egg mass (Fig. 7.1). An artificial nest containing dried eggs coated with polyester resin was constructed at the same time with similarly-placed thermocouples. Temperatures were determined with a Comark Electronic Thermometer at about noon on selected days during the incubation period. On two occasions (3-4/12/79 and 28-29/12/79, after about 25 and 60% of the incubation period) hourly readings of nest temperature were made over 24 h. Additionally, soil temperatures at 1 cm, 10 cm and 16 cm and air temperature in the grass adjacent to the nest were recorded with thermistors and a Grant continuous temperature recorder.

7.2.3 Sex Ratio of Population in Lake Bonney

To determine sex ratios in a natural population, adult tortoises were collected in Lake Bonney using three methods (baited drum traps, seine nets and by hand (Chapter 8)). This reduced sampling biases (cf. Gibbons, 1970b). Each animal was sexed using the criteria of Chessman (1978): females have relatively shorter tails and larger body size than males.

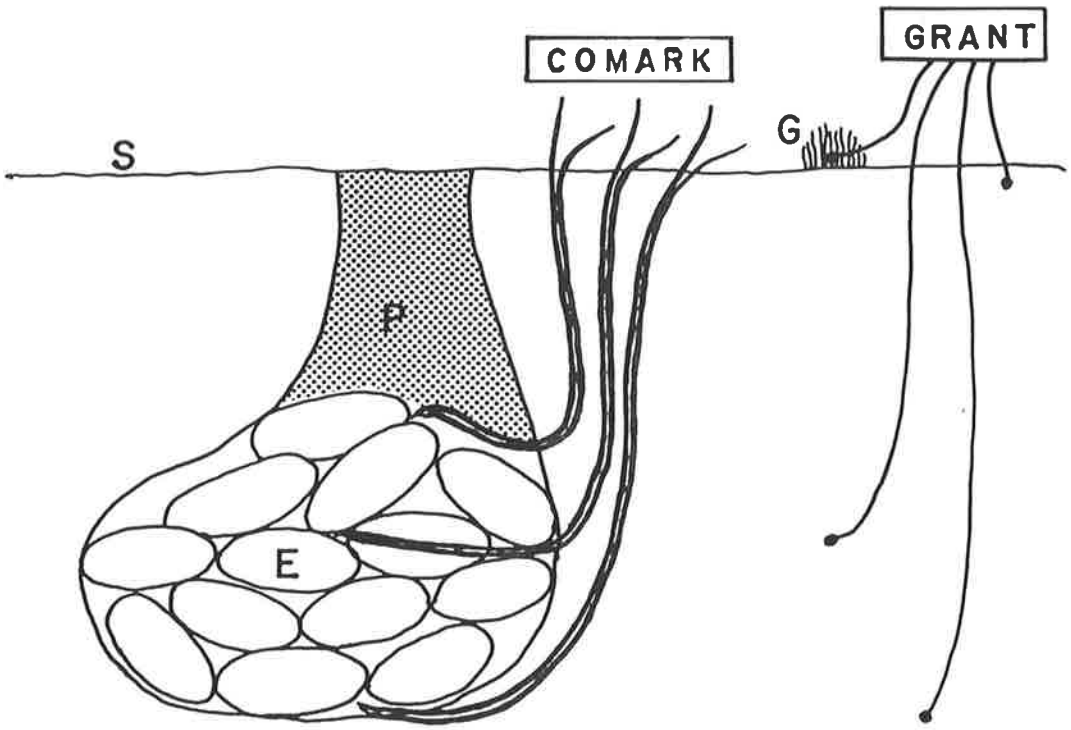


FIGURE 7.1

DIAGRAM OF NEST CHAMBER WITH EGGS (E) SHOWING POSITION OF THERMOCOUPLES.
P = nest plug, S = surface of sand, G = grass.

7.2.4 Incubation Time

Incubation times were recorded for eggs maintained at constant temperature in the laboratory. In addition, the time from oviposition to hatchling emergence on the surface was recorded in four natural nests and nineteen nests at the tortoise farm, and related to the position of the nest (i.e. in sun or shade). Temperature measurements were made on some nests.

7.3 RESULTS

7.3.1 Laboratory Experiments

Wax sections of gonads confirmed the gross examinations under the microscope, as in other species (Risley, 1933; Yntema, 1981; Ferguson & Joanen, 1982). As there is no significant difference between sex ratios of hatchlings incubated at different water potentials ($X_2^2 = 2.775$, $p>0.10$) the results are pooled at each temperature. A sex ratio not significantly different from unity occurred at all temperatures except 30°C where the ratio was biased towards males ($p<0.01$) (Table 7.1). The sex ratio of all temperatures combined is not significantly different from 1 ♂: 1 ♀ ($p>0.10$).

TABLE 7.1

Temp °C	No. Hatchlings	% Hatched	No. ♂	No. ♀	% ♂	% ♀	X_1^2	Significance
25	20	83	13	7	65	35	1.80	NS
26	19	79	7	12	37	63	1.32	NS
28	20	83	10	10	50	50	0.00	NS
30	23	96	18	5	78	22	7.35	$p<0.01$
32	18	75	10	8	56	44	0.22	NS
Combined Results	100	83	58	42	58	42	2.56	NS

Sexes of hatchling *E. macquarii* incubated at different temperatures and the X^2 values and the statistical significance when compared to the null hypothesis that the sex ratio is 1 ♂: 1 ♀. NS = not significant.

Sex ratios of hatchlings from the 30°C rerun are pooled because there is no significant difference between trials and between incubation substrate water potentials ($X_2^2 = 0.524$, $p>>0.10$ and $X_4^2 = 1.286$, $p>>0.10$). The sex ratio of 58 ♂: 53 ♀ is not significantly different from 1 ♂: 1 ♀

(X_1^2 = 0.225, p>>0.10). When added to those of the first 30°C trial the result is also not significantly different from 1 ♂: 1 ♀ (X_1^2 = 1.54, p>0.10).

Of the six eggs incubated at 20°C two were females, one was male and the other three were dissected at embryonic stages too young for sex determination.

7.3.2 Field Nest Temperatures

Mean temperatures in natural nests over 24 h, with air and soil temperatures (Figs. 7.2, 7.3) show that:

(1) nest temperatures are buffered against the extremes of temperature on the soil surface. On 3-4/12/79 the air temperature fluctuated more than 30°C (19.8 - 50+°C) compared to 12.0°C (15.2 - 27.2°C) in the centre of the nest. On 28-29/12/79 the values were 24.6°C (22.2 - 46.8°C) and 7.4°C (25.6 - 33.0°C).

(2) temperatures of the soil at the depth of the eggs were higher than the eggs on both occasions, particularly so on 3-4/12/79.

(3) temperatures in the artificial nest were similar to those of the real nests, and lower than sand temperatures (Table 7.2).

Variation in spot temperature readings (Fig. 7.4) is due in part to shading of the nests (Fig. 7.5). Spot temperature measurements show a general rise in nest temperature throughout incubation until the warmest nests hatch (Fig. 7.5). Declining temperatures thereafter are due to the warmest nests, those in full sun, being eliminated from the calculation of the means. The maximum increase in temperature during incubation was 15.2°C for a nest in full sun at the tortoise farm in 1979-80 (Fig. 7.5).

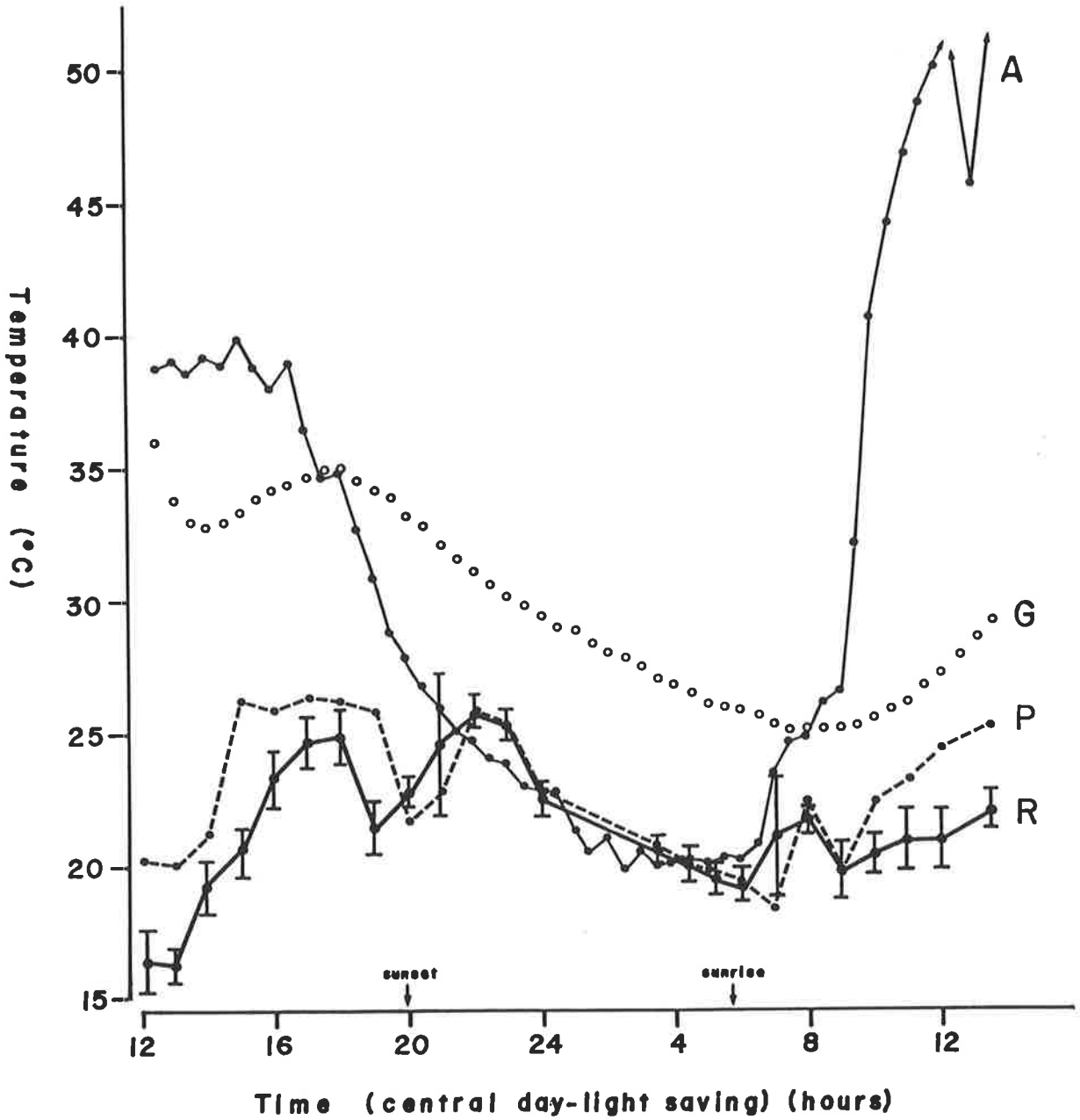


FIGURE 7.2 TEMPERATURES AT LAKE BONNEY 3-4/12/79, EIGHTEEN DAYS INTO THE INCUBATION PERIOD.

A = air temperature in the grass less than 1 cm above the sand.

G = soil temperature at 10 cm depth, which is the depth of the middle of an egg mass.

P = temperature in the middle of an artificial nest made of plastic-coated eggs

R = mean temperature in the middle of two real nests. Bars represent the actual temperatures in each nest.

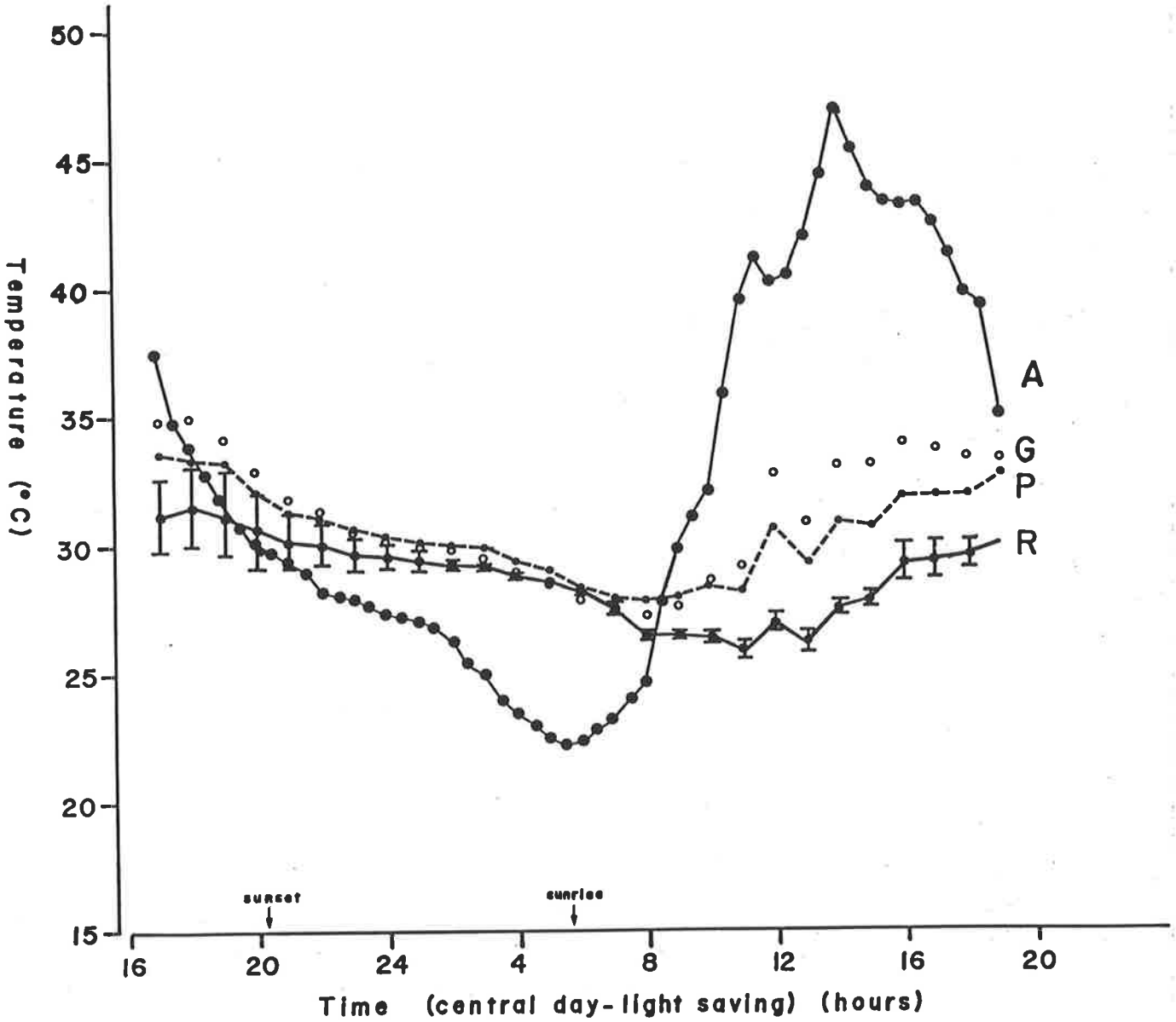


FIGURE 7.3 TEMPERATURES AT LAKE BONNEY 28-29/12/79. SYMBOLS AS IN FIGURE 6.2 EXCEPT THAT G WAS MEASURED IN THE SAME WAY AS P AND R, (APPROXIMATELY 3 WEEKS PRIOR TO HATCHING).

TABLE 7.2 MEAN, MAXIMUM AND MINIMUM TEMPERATURES (^{o}C) RECORDED HOURLY FOR 24 h TWICE DURING THE 1979-80 NESTING SEASON ON TOP OF (On), IN THE MIDDLE OF (In) AND BENEATH (Under) THE EGG MASS. 10 cm AND 16 cm ARE EQUIVALENT DEPTHS TO IN AND UNDER MEASUREMENTS

Date	Air Temp in Grass	Control Nest			Nest 5			Nest 6			Soil		
3-4/12/79		On	In	Under	On	In	Under	On	In	Under	1 cm	10 cm	16 cm
Mean	29.8	23.9	22.5	22.0	23.3	22.3	21.4	22.9	20.5	20.0	33.7	29.8	27.7
± SD	± 8.8	± 4.3	± 2.6	± 2.4	± 3.1	± 2.7	± 2.5	± 3.8	± 2.5	± 2.4	±10.7	± 3.6	± 1.2
Max	50.0	31.1	26.5	25.8	28.8	27.2	25.7	29.5	25.1	24.5	50 +	34.8	29.2
Min	19.8	17.3	18.3	18.1	19.4	16.9	15.3	17.6	15.2	14.5	22.1	25.2	25.9
Difference	30.2	13.8	8.2	7.7	9.4	10.3	10.4	11.9	9.9	10.0	27.9+	9.6	3.3
28-29/12/79													
Mean	31.6	31.0	30.2	29.7	28.3	28.2	27.7	30.9	29.2	28.6	32.2	30.8	29.9
± SD	± 7.4	± 3.0	± 1.8	± 1.6	± 1.3	± 1.3	± 1.4	± 3.2	± 2.2	± 2.1	±10.2	± 2.4	± 1.7
Max	46.8	35.8	33.6	32.5	30.6	30.0	29.3	37.9	33.0	32.0	51.9	35.0	32.5
Min	22.2	26.7	27.8	27.1	26.4	25.8	25.1	26.3	25.6	25.2	19.3	27.4	26.7
Difference	24.6	9.1	5.8	5.4	4.2	4.2	4.2	11.6	7.4	6.8	32.6	7.6	5.8

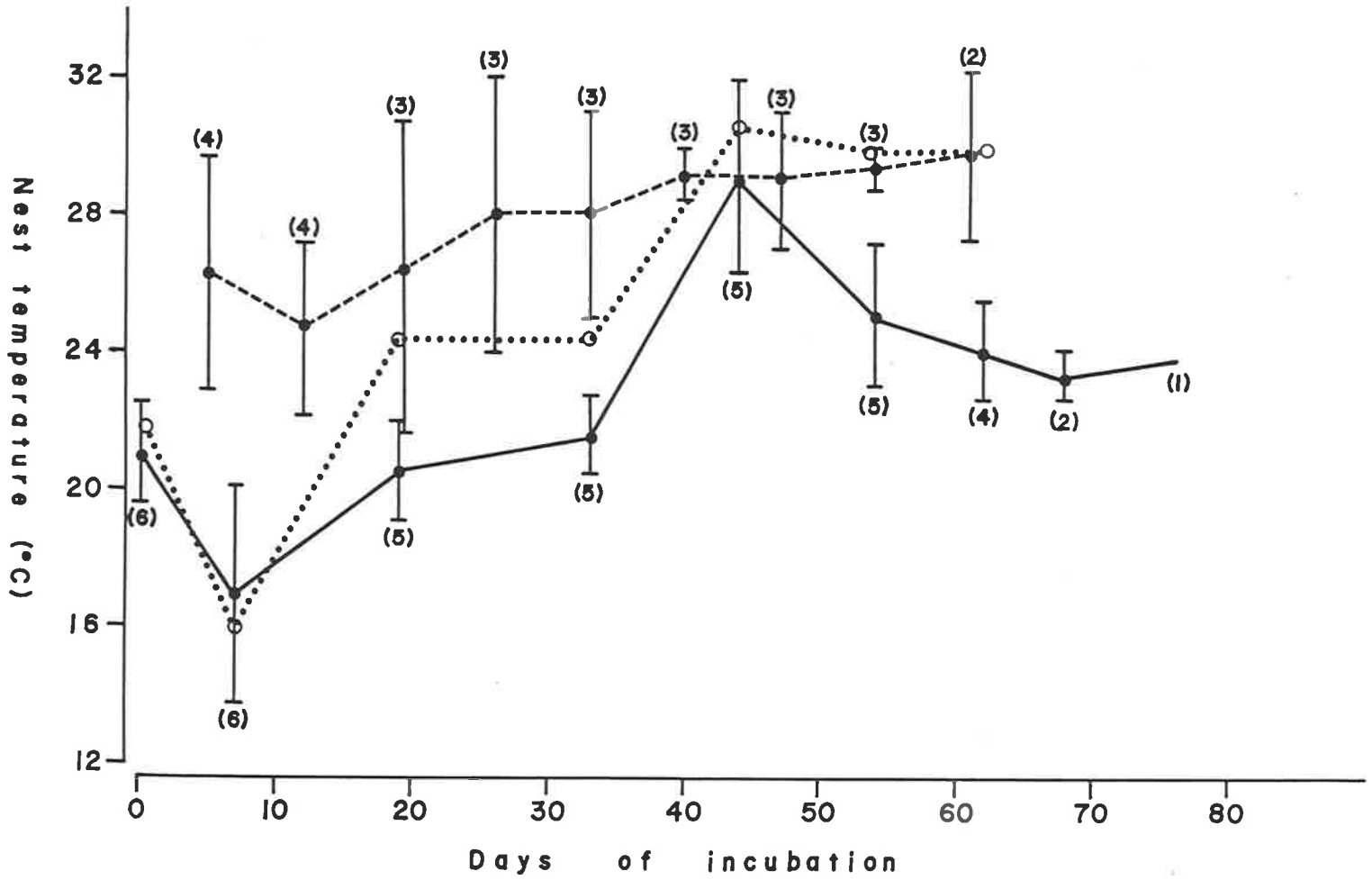


FIGURE 7.4 MEAN TEMPERATURES (^{o}C) MEASURED IN SAND AT EGG DEPTH IN 1978-79 (DASHED LINE) AND IN MIDDLE OF EGG MASS IN 1979-80 (SOLID LINE). BARS ARE ONE STANDARD DEVIATION AND NUMBERS IN PARENTHESES ARE NUMBERS OF NESTS MEASURED. DOTTED LINE REPRESENTS TEMPERATURES MEASURED IN THE ARTIFICIAL NEST IN 1979-80.

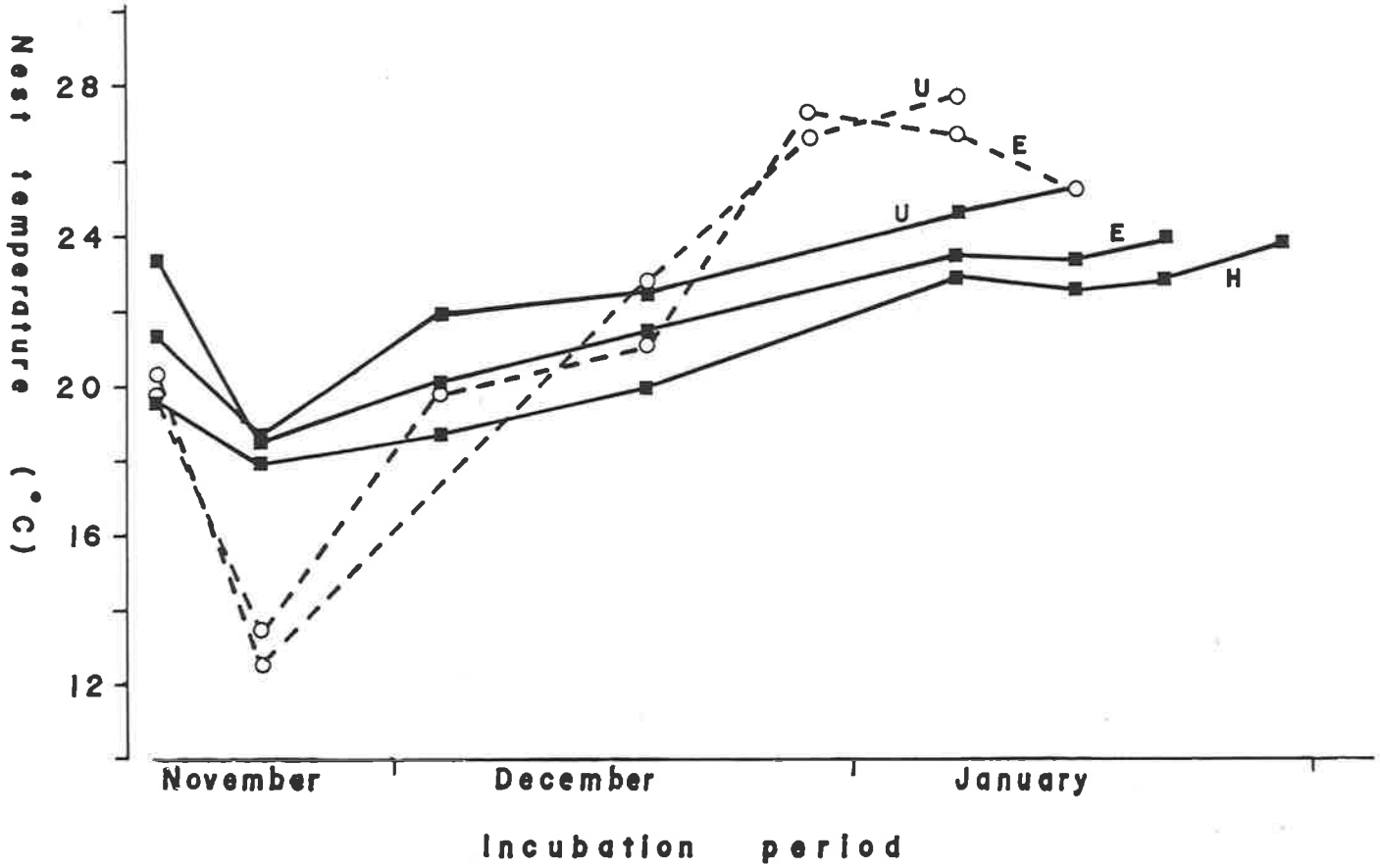


FIGURE 7.5 SPOT NEST TEMPERATURES (°C) IN MIDDLE OF EGG MASS AT ABOUT MIDDAY THROUGHOUT INCUBATION PERIOD 1979-80.
(U = nest in direct sun, E = in semishade or dappled sunlight, H = in shade).

Mean minima and maxima were 24.6 ± 2.5°C and 29.8 ± 2.5°C in 1978-9 and 16.9 ± 3.2°C and 27.0 ± 0.5°C in 1979-80.

Eggs also experience wide daily temperature fluctuations which are greater early in the season (maximum measured was 10.4°C; Fig. 7.2) than later (maximum 7.4°C; Fig. 7.3). Within a nest there are marked differences (up to 5.9°C) in the temperatures experienced by different eggs (Table 7.2). The top eggs experience greater temperature fluctuations than the bottom eggs (Table 7.2) and are warmer than the bottom eggs for more than 75% of the day (Fig. 7.6). When the bottom eggs are warmer than the top eggs the difference is much less than in the reverse situation.

In 14 nests at the tortoise farm in 1978-9 the top layer of eggs in four died late in development. Excessive heat is thought to be responsible because the maximum air temperature exceeded 40°C on five days prior to hatching including two days over 45°C (records from Weather Bureau, Renmark and Loxton). These nests were not shaded.

The temperature of soil at the depth of eggs in nests of *E. macquarii* is generally warmer than the temperature in the centre of nests (Figs. 7.2, 7.3). On one occasion, prior to dawn, the soil was cooler than the eggs (Fig. 7.3).

7.3.3 Adult Sex Ratios

Two hundred and five *E. macquarii* were caught in Lake Bonney. Two were seine netted, six caught by hand and the rest were caught in baited drum traps. Eleven were juveniles or subadults that could not be sexed with certainty. The rest were 69 ♂: 125 ♀. This is significantly

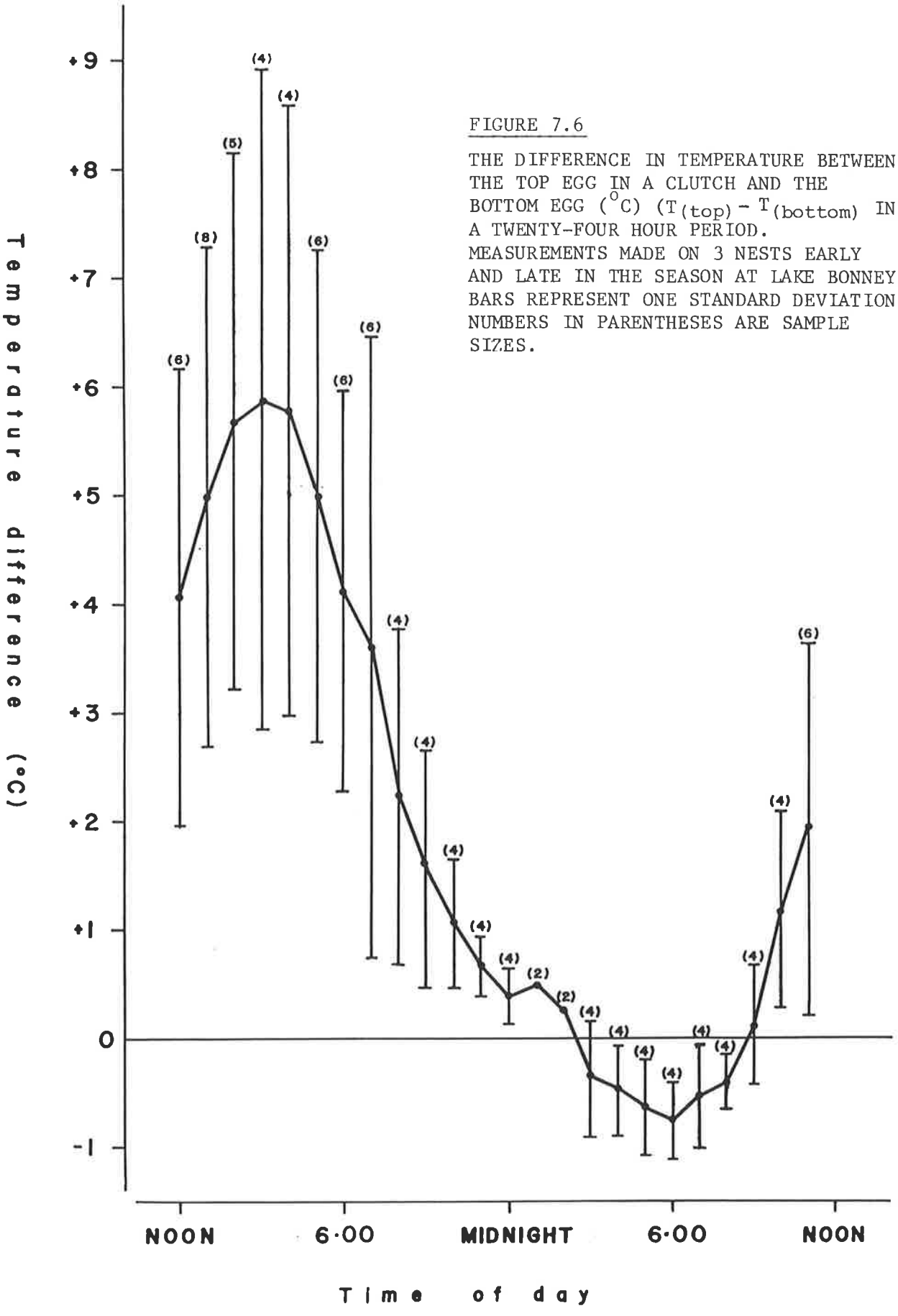


FIGURE 7.6

THE DIFFERENCE IN TEMPERATURE BETWEEN THE TOP EGG IN A CLUTCH AND THE BOTTOM EGG (^{o}C) ($T_{(top)} - T_{(bottom)}$ IN A TWENTY-FOUR HOUR PERIOD. MEASUREMENTS MADE ON 3 NESTS EARLY AND LATE IN THE SEASON AT LAKE BONNEY. BARS REPRESENT ONE STANDARD DEVIATION. NUMBERS IN PARENTHESES ARE SAMPLE SIZES.

different from 1 ♂:1 ♀ (X^2_1 = 16.165, $p<0.001$).

7.3.4 Incubation Times

As substrate water potential does not alter incubation times, water potential was eliminated as a variable and results from all eggs incubated at any one temperature are combined for the analysis. Higher incubation temperatures result in reduced incubation times (Fig. 7.7) with the temperature of incubation (T) being linearly related to the inverse of the incubation time (I) (Fig. 7.8) by

$$(I)^{-1} = 0.00121\ T - 0.0155 \quad (r^2 = 0.979,\ n = 6) \qquad (7.1).$$

Three of the six eggs transferred from 20 to 30°C after 91 days of incubation pipped the shell after 114 days (i.e. 91 days at 20°C and 23 days at 30°C) (Fig. 7.7); two others died sometime before day 114, possibly before being transferred to 30°C and the embryos were too decayed for dissection. The sixth egg pipped but died before emerging from the shell. The eggs dissected at days 165 and 200 were alive and well developed with a large external yolk. The egg dissected at day 229 was dead and at the same embryonic stage as those killed on days 165 and 200.

The time from oviposition to hatchling emergence on the surface is related to two factors: (1) date of nesting and (2) position of the nest (Fig. 7.9). It is assumed that the hatchlings begin to dig to the surface soon after hatching and that the time taken to reach the surface is similar for all nests. Therefore differences in the time taken for hatchlings from different nests to emerge on the soil surface should reflect incubation times. However, this is not fully substantiated. Eggs deposited early in the season take almost 1.5 times as long to emerge as those deposited late in the season. Nesting peaks in the third week of November and incubation times of nests deposited then are intermediate. Nests con-

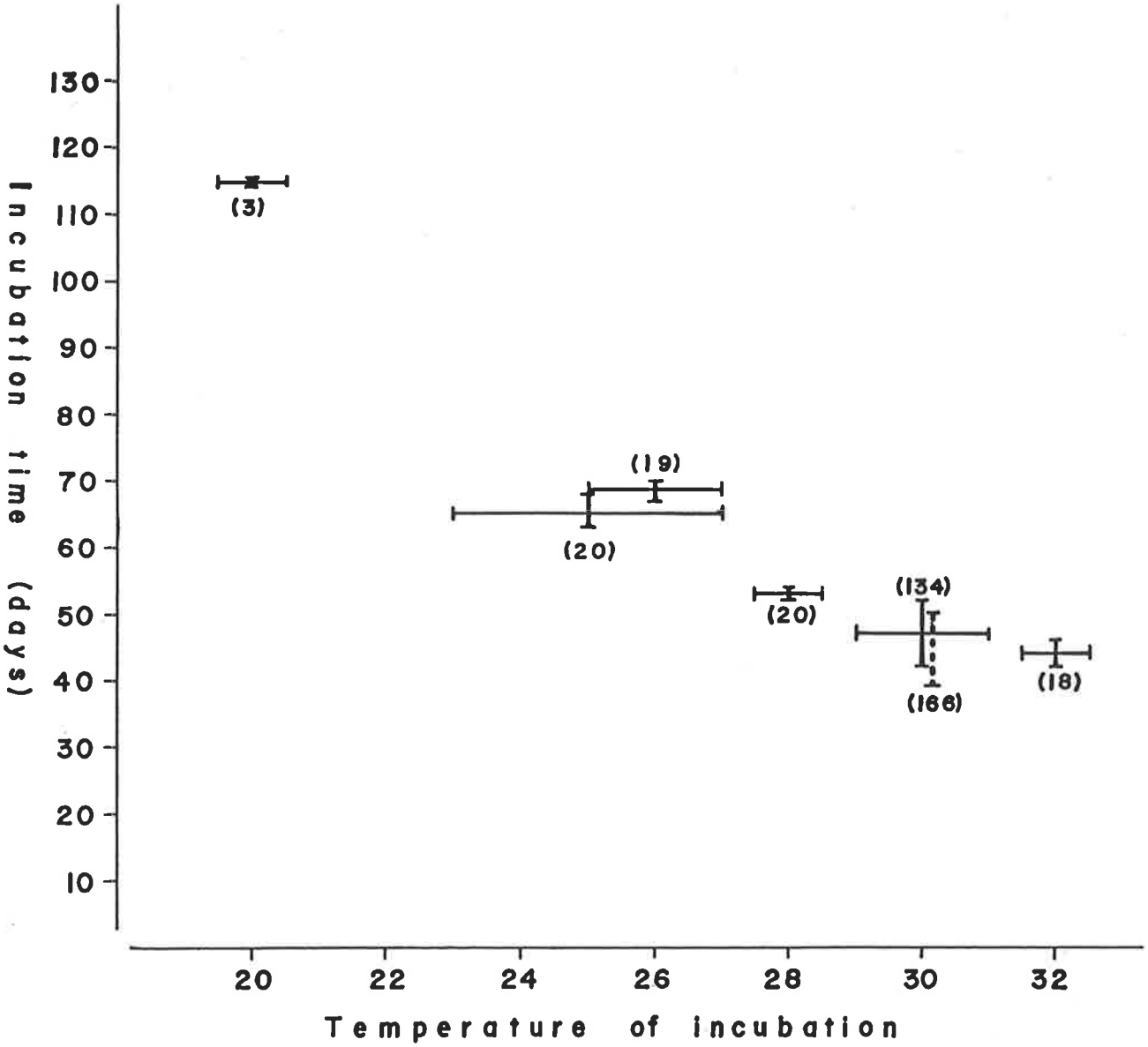


FIGURE 7.7

INCUBATION TIMES AT DIFFERENT TEMPERATURES. HORIZONTAL BARS REPRESENT TEMPERATURE FLUCTUATION DURING INCUBATION; VERTICAL BARS REPRESENT RANGE OF INCUBATION TIMES. NUMBERS IN PARENTHESES ARE SAMPLE SIZES. VALUE AT 20°C REPRESENT EGGS CHANGED FROM 20°C TO 30°C AFTER 91 DAYS. DASHED VERTICAL BAR AT 30°C REPRESENTS RANGE OF HATCHING TIMES RECORDED BY GOODE & RUSSELL (1968).

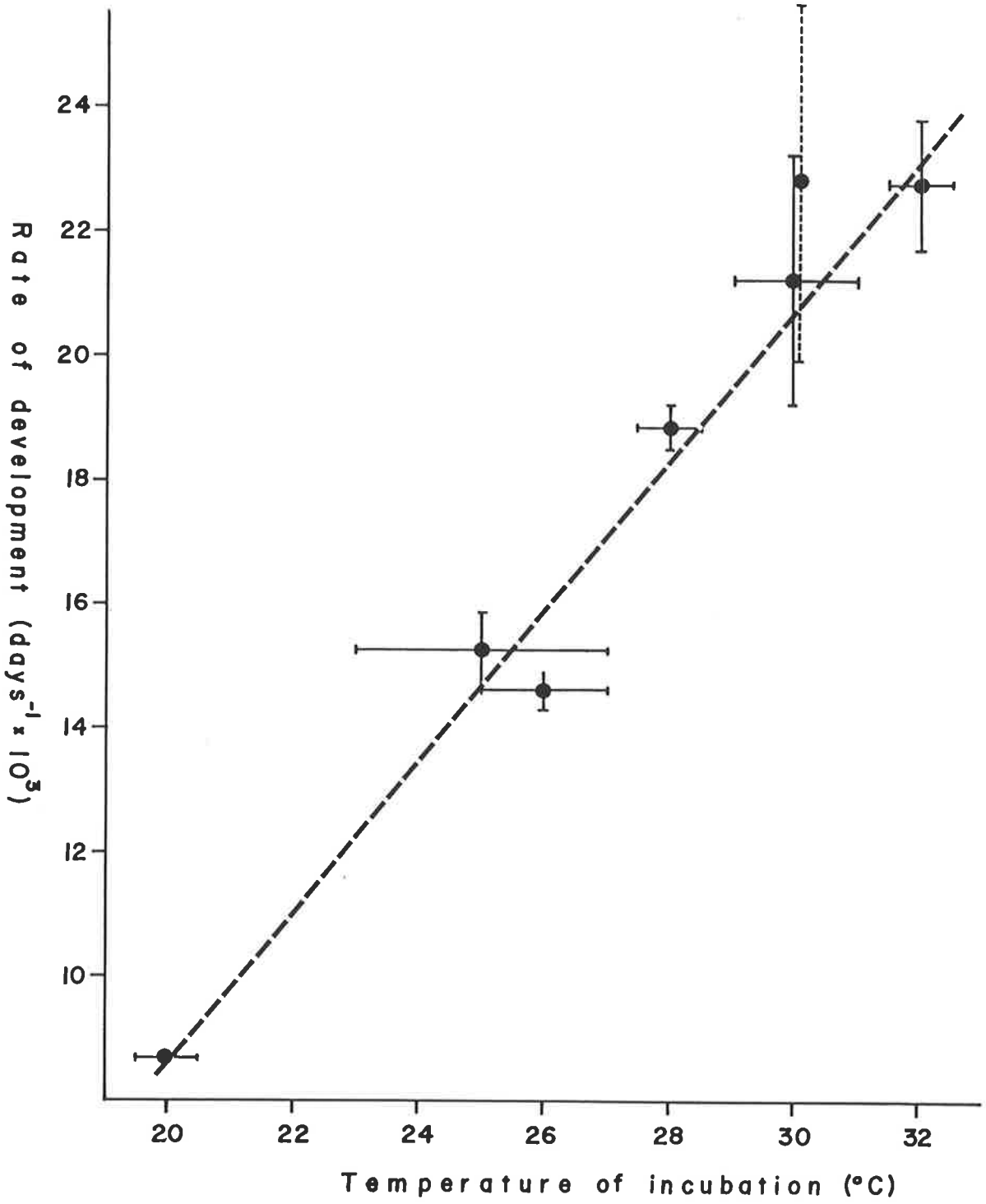
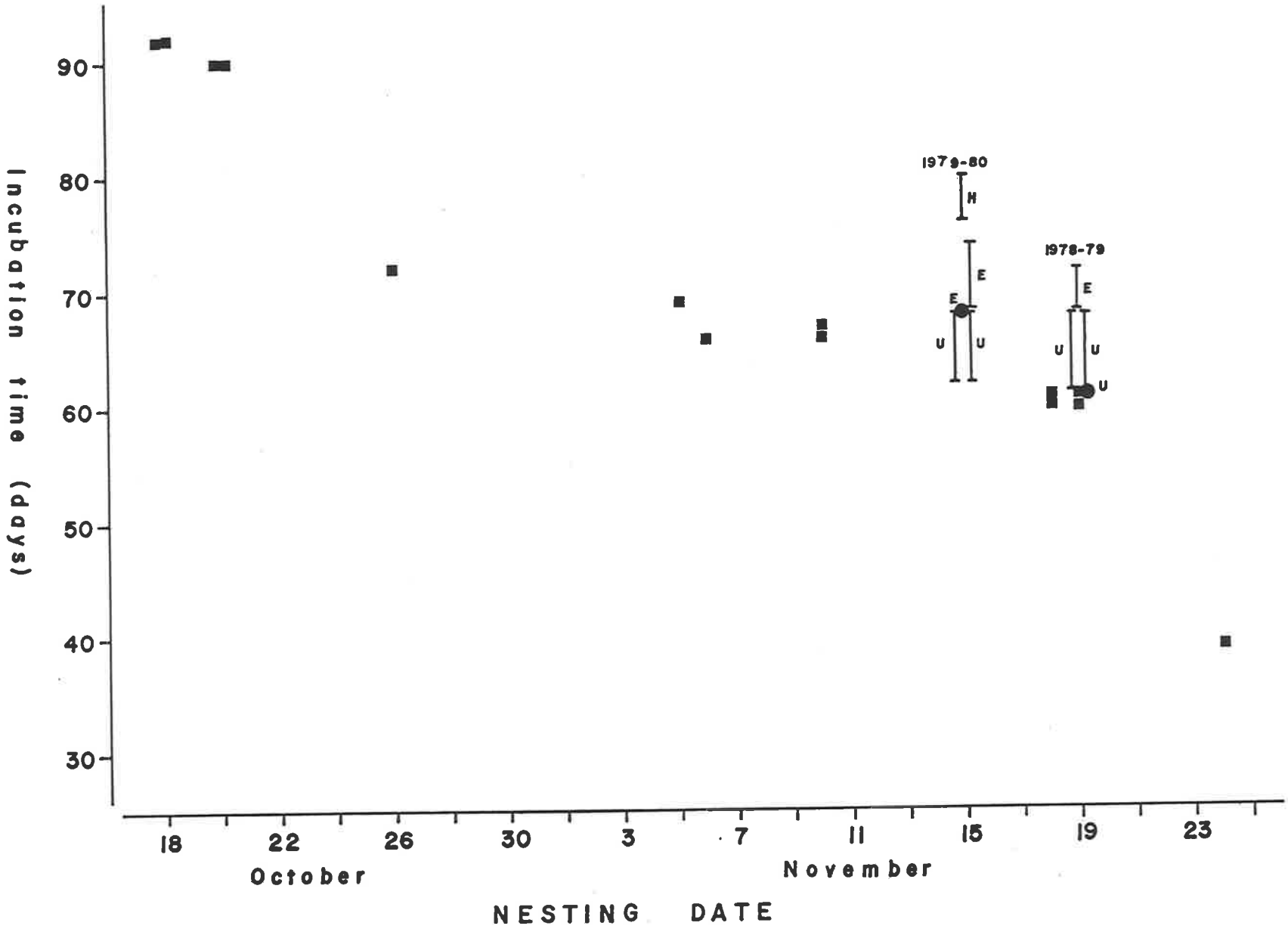


FIGURE 7.8 RATE OF DEVELOPMENT (= INVERSE OF INCUBATION TIME (days^{-1} x 10^3)) RELATED TO THE TEMPERATURE OF INCUBATION ($^{\circ}$C). HORIZONTAL BARS REPRESENT TEMPERATURE FLUCTUATIONS DURING INCUBATION. VERTICAL BARS REPRESENT THE RANGE OF INCUBATION TIMES. REGRESSION EQUATION 6.1 IS FITTED.

FIGURE 7.9 Nesting dates vs incubation times for 14 nests at a commercial tortoise farm (■) in 1978-79. Bars and ● represent the hatching times of 4 nests in 1978-79 and 5 nests in 1979-80 (2 and 2, and 2 & 3 from Lake Bonney and the tortoise farm respectively) selected to show the effects of shading on hatching times in the field.
(H = totally shaded; E = semishaded; U = in full sun.)


Incubation time (days)
90
80
70
60
50
40
30
1979-80
H
E
E
U
U
1978-79
E
U
U
U
18
22
26
30
3
7
11
15
19
23
October
November
NESTING DATE

structed on the same day show a difference in emergence time of between 10 and 18 days depending on the position of the nest. Nests in full shade take longer to hatch than those in the sun.

Q_{10} of incubation was calculated by:

$$\log Q_{10} = {}^{10}(\log T_1 - \log T_2)\ (t_2 - t_1)^{-1}$$

where $T_{(2)}$ is the incubation time at temperature $t_{(2)}$ and $T_{(1)}$ is incubation time at $t_{(1)}$. The Q_{10} falls with increasing temperature (Table 7.3).

TABLE 7.3 Q_{10} OF DEVELOPMENT AT DIFFERENT TEMPERATURE INTERVALS

Temperature (^{o}C)	Q_{10}
20 - 25	3.06
25 - 28	2.03
28 - 30	1.82
30 - 32	1.39

7.4 DISCUSSION

7.4.1 Field Nest Temperatures

The eggs of *E. macquarii* are deposited in relatively shallow nests (Chapter 6) and are thermally influenced in a similar way to other species with shallow nests, e.g. *Pseudemys scripta* (Cagle, 1939, 1950), *Chelodina expansa* and *C. longicollis* (Goode & Russell, 1968), *Podocnemis unifilis* (Medem, 1969) and *Malaclemys terrapin* (Burger, 1976). All buried eggs experience less variation in temperature than the soil surface and the influence of diurnal variations decreases with nest depth. Thus eggs in shallow nests undergo considerable diurnal fluctuations, up to 18°C in artificial nests constructed to resemble natural nests of *Emys orbicularis* (Pieau, 1982). In contrast, the depth of sand covering the deeply buried eggs of marine turtles, such as *Chelonia mydas*, insulates them from the marked diurnal fluctuations in sand surface temperatures (Hendrickson, 1958; Bustard & Greenham, 1968). *Geochelone elephantopus*, which lays eggs at depths intermediate between those of marine turtles and species like *E. macquarii*, experience lesser diurnal temperature fluctuations than eggs at shallower depths (MacFarland & Reeder, 1975).

The amount of variation in nest temperatures also depends on a number of other factors including the thermal conductivity of the soil, shading of the nest or its orientation towards the sun and fluctuations in air temperatures above the nest. The eggs of the tropical rainforest species, *Podocnemis unifilis*, which occurs in an environment with relatively small temperature variations, experience smaller diurnal temperature fluctuations, about 1°C (Medem, 1969; Alho & Pádua, 1982), than species which live in a temperate environment e.g. in *Malaclemys terrapin* (Burger, 1976) and *E. macquarii* diurnal fluctuations are about 10°C.

The temperature of the nest containing plastic eggs was always close

to that of the real nests of *E. macquarii*, and usually within the range of real nest temperatures (Figs. 7.2, 7.3) which indicates that the eggs do not increase the temperature of the nest significantly with metabolic heat. In contrast, the large clutches of turtle eggs (2.9 to 7.1 kg. Ackerman, 1980) significantly increase their temperature (up to 7°C) with metabolic heat (Hendrickson, 1958; Carr & Hirth, 1961; Bustard & Greenham, 1968; Bustard, 1971a; Raj, 1976). The nests of *Malaclemys terrapin*, which are only one-third the size of those of *E. macquarii*, are 2 - 7°C above the adjacent sand due to metabolic heating (Burger, 1976). It is difficult to explain this large increase in such small clutches of eggs when no increase was recorded in *E. macquarii* during incubation and the temperature increase in the large clutches of marine turtles is of the same magnitude. The differences may be related to the insulative properties of the soils in question but their thermal conductances are unknown.

7.4.2 Incubation Time

There are many records of incubation time at single controlled temperatures (summarized by Ewert (1979) and Miller and Limpus (1981)) but very few reports on the rate of incubation in a range of controlled temperatures (e.g. Dodge *et al.*, 1978; Yntema, 1978).

With incubation times at different temperatures for 57 species of 8 families (Ewert, 1979), four species of marine turtles (Miller & Limpus, 1981) and my results for *E. macquarii* (Fig. 7.10) I calculated the relationship between the temperature of incubation (T) in the range 20 - 33°C and incubation time (I) to be:

$$(I)^{-1} = 0.00109\ T - 0.0163 \quad (r^2 = 0.411,\ n = 167) \qquad (7.2).$$

The slope of equation 7.1 is not significantly different from that of equation 7.2 ($t_{(2),169} = 0.32$, $p>0.50$). Several species included in the

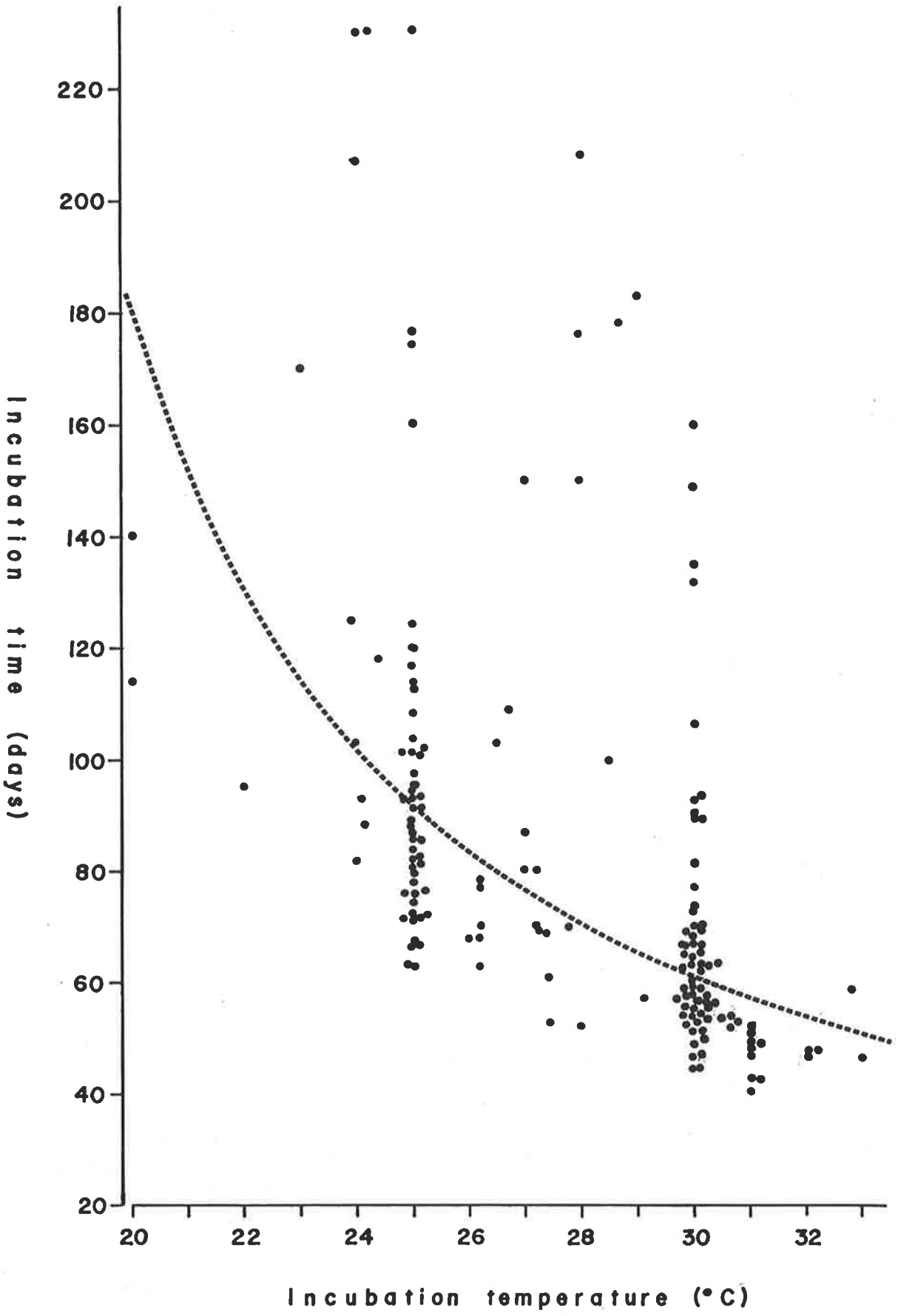


FIGURE 7.10 THE RELATIONSHIP BETWEEN INCUBATION TIME AND TEMPERATURE FOR 57 SPECIES FROM EWERT (1979), FOUR SPECIES FROM MILLER & LIMPUS (1981) AND FOR *E. MACQUARII*. EQUATION 7.2 IS FITTED.

calculation of the equation, do not fit it very well and their inclusion reduces the coefficient of determination. They include the family Testudinidae and the species *Chelodina expansa*, *Elseya dentata* and *Kinosternon leucostomum* (Ewert, 1979).

The rate of growth of embryonic *Chelydra serpentina*, measured as the amount of time to reach certain embryonic stages, is much more rapid at the beginning of incubation at 30°C than at 20°C but this difference declines throughout the incubation period (Yntema, 1968, 1978). Development proceeds at the same rate during the last three weeks of incubation at 20 and 30°C.

Similar to eggs of other chelonians (Yntema, 1978; Pieau, 1982) eggs of *E. macquarii* cannot complete incubation at 20°C but can survive at least 91 days and then successfully complete incubation at 30°C.

7.4.3 Temperature-dependent Sex Determination

The temperature range used in this experiment (20 - 32 C) covers all the known threshold temperatures. As all temperatures tested gave an equal sex ratio the conclusion must be that sex is determined independently of temperature in *E. macquarii*. *E. macquarii* does not have heteromorphic sex chromosomes (Bull & Legler, 1980) and the mechanism of sex determination is unknown. Chromosomal sex determination is likely because other species of chelonians without heteromorphic sex chromosomes have a ZZ/ZW or XX/XY chromosomal system (Engel *et al.*, 1981).

Although TSD has been known in reptiles for some time (Bull, 1980) and its importance in the husbandary and conservation of economically important species such as marine turtles and endangered species such as

Geochelone elephantopus has been discussed (Swingland, 1979; Mrosovksy & Yntema, 1980; Morreale *et al.*, 1982) very little is known about its selective advantage or mechanism. The selective advantage of TSD has been discussed in terms of fitness of resulting progeny (Bull, 1980, 1981). This was initially suggested by Charnov and Bull (1977) as a general theory for any environmental sex determination, of which TSD is an example. In general terms, this theory assumes that selection will favour the development of an above average male over a below average female in one set of environmental conditions and an above average female over a below average male in another set of environmental conditions. (An above average individual is one that contributes more genes to the next generation.) However, all the examples given by Charnov and Bull are species in which sexual maturity and the advantage gained by environmental sex determination is realised very soon after the environmental stimulus is exerted and all are invertebrates (e.g. some parasitic nematodes, some parasitic isopods, monstrillid crustaceans and an echiurid).

Chelonian species generally reach sexual maturity many years after hatching (typically 4 - 10 years for freshwater species, e.g. Hildebrand, 1932; Tinkle, 1958; Burbidge, 1967; Gibbons, 1970c; Christiansen & Dunham, 1972; Parmenter, 1976; Cann, 1978; Chessman, 1978). The existence of environmental sex determination implies a changing or heterogeneous environment. Yet if an environmental stimulus (e.g. temperature) determines sex over only five embryonic stages during incubation (Yntema, 1979), the environment that produced that response may well have changed by the onset of sexual maturity. Chelonians have a long reproductive life which could include a variety of environmental conditions. Consequently Bull's (1980, 1981) explanation for the possible selective advantage of TSD seems unlikely. He actually states that there is no evidence to support

his model and that the aquisition of the necessary evidence may be difficult (Bull, 1980).

Female *Alligator mississippiensis* incubated at 30°C are heavier than males at 34°C and after one year of growth the females are larger and heavier than males (Ferguson & Joanen, 1982). As it is desirable for female alligators to become large and sexually mature quickly this example was cited by Ferguson and Joanen as a vertebrate species supporting the Charnov-Bull model. However the size difference between males and females attained after one year is not maintained to sexual maturity and so the evidence in support of the model is not conclusive.

Bull (1980) also presented an alternative hypothesis: that TSD may not be more advantageous than genotypic sex determination and may be, in fact, less advantageous but persists because of a lack of mutations that would allow the development of genotypic sex determination. This cannot be supported because two independent developments of heteromorphic sex chromosomes (and therefore the absence of TSD, Bull, 1980) have now been identified in chelonians (Carr & Bickham, 1981).

There is a possible selective advantage to TSD that has not yet been considered. Nests of species with TSD usually produce hatchlings of predominantly one sex (usually entirely one sex) (Bull & Vogt, 1979) which ensures outbreeding. Inbreeding is regarded as disadvantageous (Kalmus & Smith, 1960). For some species of North American freshwater turtles mortality of eggs is high but the chance of the hatchlings from the few nests that survive reaching sexual maturity is also high (Gibbons, 1968a). It follows that a large percentage of one cohort may come from one nest, particularly in small discrete habitats such as ponds. With genotypic sex determination the sex ratio should be 1 ♂: 1 ♀ from each nest and

therefore the likelihood of inbreeding exists. On the otherhand, if all the hatchlings from one nest are one sex then mating with sibs is impossible. It is in small, discrete populations, such as ponds, that TSD will have its greatest selective advantage, this advantage being reduced with increased population size and mixing.

There is an indication that many populations of chelonians have a preponderance of females. In some species this may reflect the hatchling sex ratio and may be related to TSD (Vogt, 1980). For example, a population of *Graptemys*, which has TSD, has a skewed adult sex ratio (0.8 ♀: 0.2 ♂, Vogt, 1980; Vogt & Bull, 1982a) but *Trionyx*, which does not have TSD, has an equal adult sex ratio (Vogt & Bull, 1982b). However, *E. macquarii*, which does not have TSD, and has an equal sex ratio at hatching in the laboratory, has a skewed adult sex ratio (69 ♂:125 ♀) in the field. It is assumed that the hatchling sex ratio in the field is also equal so any deviation from this in adults must be due to other factors, such as differential mortality (Williams, 1979). Therefore any attempt to link adult sex ratios with hatchling sex ratios in species with TSD must be treated cautiously because these other factors may also be operating. One cause of apparently unequal sex ratios may be biased sampling methods (Tinkle, 1958; Ream & Ream, 1966; Gibbons, 1969; Vogt, 1979). It is not possible to determine whether the sampling methods used to collect adult *E. macquarii* in this study are unbiased.

It is interesting to speculate on the lack of TSD in *E. macquarii*. Of sixteen Cryptodiran species studied, only one lacks TSD. A further three cryptodiran species have heteromorphic sex chromosomes which are mutually exclusive with TSD (Bull, 1980). *E. macquarii* is the only pleurodiran species to have been examined. It is possible that pleurodires do not have TSD and that it evolved in the cryptodiran lineage with

subsequent re-establishment of genotypic sex determination in some species. Such an hypothesis could be tested by examining more species. Alternatively TSD could have evolved many times in chelonians; TSD may have independent origins in chelonians, and lizards and alligators because higher temperatures result in females in the Chelonia and males in lizards and alligators.

8

PREDATION ON EGGS

8.1 INTRODUCTION

In the early stages of this study it was realised that the introduced red fox, *Vulpes vulpes*, was taking a very high percentage of Chelid tortoise eggs from nests on the River Murray in South Australia. Although predation by foxes on tortoise eggs had been noted previously (Parmenter, 1976; Green, 1980), no detailed study of the effect of such predation had been undertaken.

Three species of tortoise inhabit the Murray River system in South Australia where foxes abound. *Emydura macquarii* and *Chelodina longicollis* are abundant whereas *Chelodina expansa* is less common; none of these species is confined to the River Murray (Cann, 1978; Legler, 1978; Cogger, 1979). Foxes are virtually absent from the Cooper Creek area where a fourth, as yet undescribed, *Emydura* species occurs (Cann, 1978).

The rate of predation on the nests of River Murray tortoises, particularly *E. macquarii* and *C. longicollis*, was quantified from observations at various sites in South Australia. Comparisons were made of the age structure of the population of *E. macquarii* in the Murray and the *Emydura* species in the Cooper Creek, species which are ecologically and morphologically similar, to establish what the structure of *E. macquarii* populations may have been like in the Murray before foxes were introduced. No information is available on the age structure of populations of *E. macquarii* in the River Murray in the past with which direct comparisons might have been made.

8.2 MATERIALS AND METHODS

8.2.1 Survey of Nesting Sites

Major periods of nesting activity are easily predicted because they occur during or just after late spring or early summer rains. Although the tortoises may lay their eggs at any time of the day or night, if it is raining or heavily overcast, they seem to prefer night. Three nesting sites were examined throughout the 1979-80 nesting season (Table 8.1): the Barmera site which included the Lake Bonney shoreline, McIntosh Canal and Loveday Swamp; the Monteith site situated 8km south-east of Monteith; and the Milang site on the shores of Lake Alexandrina (Fig. 2.1). The Barmera and Monteith sites were examined again after nesting had begun in the 1980-81 season. A survey of the Cooper Creek was conducted about four months after the end of the nesting season in 1981.

Nesting sites were thoroughly searched for evidence of nesting. Fresh nests were easily detected because excavated soil placed into the nest cavity after oviposition was different in colour from the surrounding ground, and the adjacent vegetation was disturbed. Often pieces of grass protruded from the nest plug and tracks were sometimes present. Many intact nests were removed to the laboratory for other studies (n = 7) or protected by wire mesh (n = 3) to allow other field studies. Eggs remaining in partly destroyed nests were collected. Positions of destroyed nests were noted and marked to avoid recounting at a later date. The likely predator was determined from all available traces. Often tracks or faeces were associated with the nest remains. Four predators were identified, each having a characteristic method of excavating and eating the eggs.

An excavated nest can be identified up to twelve months later. The

hole, spoil heap and often some of the eggshell fragments are still visible. Nests in which hatchlings have developed are virtually invisible a week after hatching because there is no discernible hole or spoil heap and eggshell is never exposed above the surface.

8.2.2 Determining Population Age-structure

The age structure of the various populations was determined from a sample of about 200 individuals of each species. In some populations of *Chrysemys picta* plastron length was found to be a useful index of age in sub-adults but not adults (Gibbons, 1968a) whereas in others it could be used for all age groups (Ernst, 1971b; Wilbur, 1975a,b). Curved plastron length, which was used as an index of age in this study was taken along the midline from the anterior tip to the centre of the anal notch. In addition the maximum carapace length and the plastron width at the junction of the hypo- and xiphi-plastrons were measured (to the nearest millimeter with a steel tape) on the *Emydura* sp. from the Cooper Creek. All animals were marked with notches filed into the marginal scutes of the carapace (Cagle, 1939; Thompson, 1982) so that recaptured animals could be recognised and not counted twice.

Three capture techniques were used to reduce errors of trap bias. However, by necessity, some techniques were used more frequently than others; some were more successful than others (Table 8.2).

1. Drum trapping. Standard two and three hooped fish traps with funnelled entrances and two metre wings and wire traps with funnelled entrances were used. Each trap was baited with sheep liver, ox liver, whole skinned rabbit, sliced whole fish or fish-based canned cat food that was suspended in the trap in a piece

of stocking. This technique was the most successful for *Emydura* species. A few *C. longicollis* and one *C. expansa* were also caught in this way.

2. Hand capture. Most of the *C. longicollis* were caught by hand in shallow swamps. Muddling, which involves feeling for tortoises with the feet (Plummer, 1979), was occasionally employed in swamps when the water was turbid. It also resulted in the capture of some *Emydura* species.

3. Seine netting. Seine netting proved to be largely unsuccessful although used with moderate success on *Emydura* in Cooper Creek.

Three *C. longicollis* were also accidentally collected with baited hooks.

Sexually mature tortoises were sexed using the method of Chessman (1978). Females grow much larger than males (Fig. 8.2) and their tails are proportionally shorter and thinner. For both species of *Emydura* immatures were recorded simply as juveniles. All juveniles are morphologically similar to females, hence there was some overlap in the sizes of individuals recorded as juveniles and others as either males or females. The onset of secondary sexual characteristics has been used in other studies to indicate the attainment of sexual maturity (Cagle, 1946, 1950; Gibbons, 1968a), although the correlation may not be reliable in all individuals (Christiansen & Moll, 1972, 1973; Cagle, 1954). The minor errors associated with this did not significantly alter the results. *C. longicollis* could not be sexed reliably so the results for males and females of this species were pooled.

All the dead tortoises found on Cooper Creek were collected and the

curved plastron length measured. Where the plastron was damaged another measurement that could be mathematically related to plastron length was measured. Dead tortoises collected by a subsequent visit to the Cooper Creek by colleagues in 1982 were included in the analysis.

8.3 RESULTS

8.3.1 Nest Predation

Nesting season and sites of *C. longicollis* and *E. macquarii* overlap to a large extent. Without excavating the eggs it is impossible to tell which species has nested. Similarly, once a nest is destroyed, the species cannot be determined from the remains so the predation rate was assumed to be equal in both species and the results were pooled (Table 8.1). It was considered likely that all intact nests were located.

At the Monteith site an adult female *E. macquarii* was found dead on the nesting beach with its head and legs eaten. Presumably it had come up to nest and been killed by foxes. At Milang all but one dead *C. longicollis* found had its head and legs missing. Several live individuals were found with legs missing.

Four nest predators were recognised. Foxes were the most prevalent, taking 93% of nests (Table 8.1). Often tracks were associated with the destroyed nest and in many instances the fox defaecated on the destroyed nest. The nest plug was completely removed and sometimes the entrance was enlarged. Only the egg contents were eaten, leaving empty shells. The golden water rat, *Hydromys chrysogaster*, was the major endemic predator, taking 1.6% of nests. None of the nests destroyed by water rats was more than three metres from the water. A narrow entrance was dug into the nest chamber. The eggs were then removed with the incisor teeth, placed on the ground, opened and the contents eaten. Sometimes the incisor marks were still visible in the discarded eggshell. Goannas took at least 0.5% of nests. Two species may have been involved, *Varanus varius* and *V. gouldii*, as both are known to occur along the Murray. This estimate may be slightly low because they excavated the nest widely and ate the entire egg, shell and all. Hence it was difficult to identify

TABLE 8.1 NEST PREDATION DATA COLLECTED AT THE THREE STUDY SITES ON THE RIVER MURRAY

Locality	Date	Probable Predator				Partly Destroyed	Undisturbed	Total
		Fox	Goanna	Raven	*Hydromys*			
Barmera	15. xi.79	42	2	2		2	9	57
Barmera	21. xi.79	1						1
Monteith	21. xi.79	19						19
Monteith	13.xii.79	11				1		12
Barmera	28.xii.79	3						3
Barmera	8. i.80	10			3			13
Milang	11. i.80	2					1	3
Barmera	30. i.80	69						69
Barmera	27. ii.80	2						2
Monteith	15. iv.80	53						53
Monteith	16. iv.80	5					2	7
Barmera	16. iv.80						1	1
Barmera	18.xii.80	39			3			42
Monteith	18. i.81	87						87
Totals		343	2	2	6	3	13	369
Percentage			.54	.54	1.63			
		93.0		2.7		.8	3.5	100%

such nests unless claw marks were present. Two nests had been dug out by ravens (*Corvus coronoides*). Both were in loose sandy soil. The nests were widely excavated and raven footprints were numerous. In one case an eggshell was found in a nearby tree. Only the egg contents were eaten.

Death of eggs caused by other factors was rare and generally only affected part of the clutch. The only other cause of death identified was excessive heat that killed the top eggs of some clutches in an exceptionally hot year. One egg containing fly larvae was found. Fly infested eggs have also been reported in other species (Vestjens, 1969; Vogt, 1981). Drowning was not a factor affecting eggs in the areas examined. Some apparently infertile eggs were found in most nests of *E. macquarii* and have been reported in many other species (e.g. Vestjens, 1969).

No destroyed nests were seen anywhere along the Cooper. This indicated that nest predation there is rare. One eggshell fragment was found blown under a log but the nest from which it came could not be found. For eggshell to be on the surface the nest must have been dug up. There was no indication of the predator involved but it could not have been a goanna otherwise the eggshell would have been eaten too.

8.3.2 Population Age Structure

Emydura was more easily caught in drum traps than *Chelodina* (Table 8.2). Because the same traps were used to collect *E. macquarii* in the Murray as *Emydura* species in the Cooper, and because they reach sexual maturity at the same size (age) (Fig. 8.2), the age structures of these populations were compared directly to each other (Fig. 8.2) whereas *C. longicollis* was considered separately (Fig. 8.3). In comparing the two *Emydura* species the null hypothesis assuming that the two populations

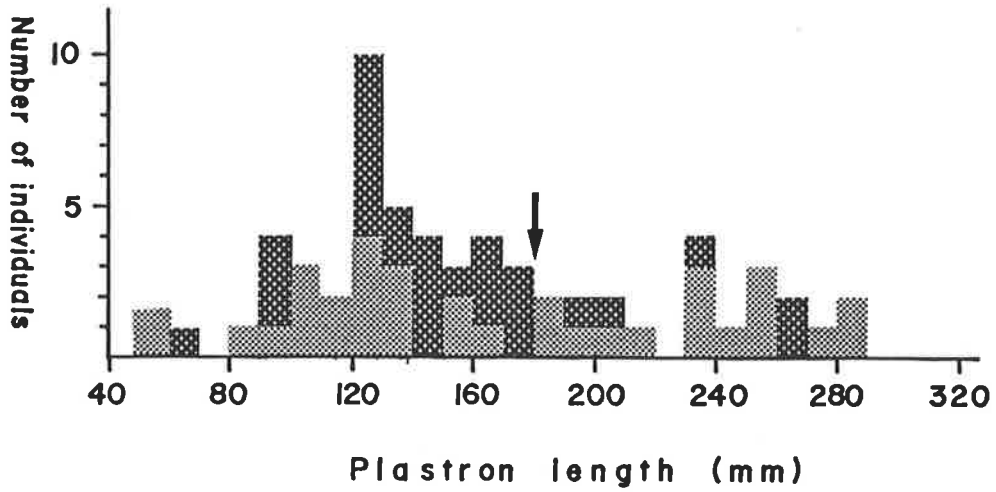


FIGURE 8.1 SIZE CLASSES OF THE DEAD *EMYDURA* SP. COLLECTED FROM THE COOPER CREEK. LIGHT STIPPLE REPRESENTS SPECIMENS COLLECTED IN 1981 (n = 33); DARK STIPPLE ARE SPECIMENS COLLECTED IN 1982 (n = 28). ARROW INDICATES THE APPROXIMATE ONSET OF SEXUAL MATURITY.

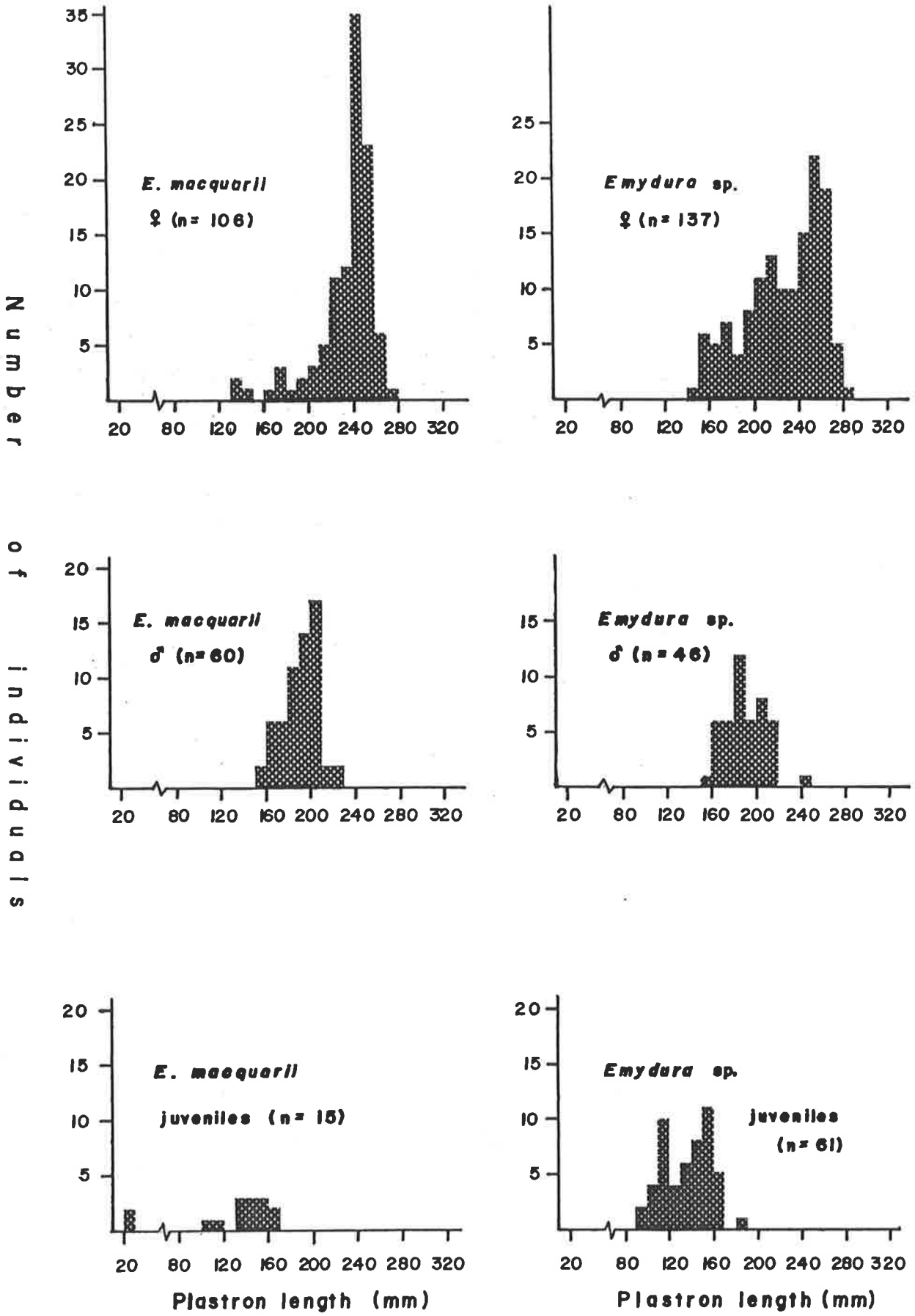


FIGURE 8.2 SIZE CLASSES OF ALL THE *E. MACQUARII* COLLECTED IN THE RIVER MURRAY AND *EMYDURA* SP. COLLECTED IN THE COOPER CREEK. SEXES AND JUVENILES WERE PLOTTED SEPARATELY FOR EASY COMPARISON.

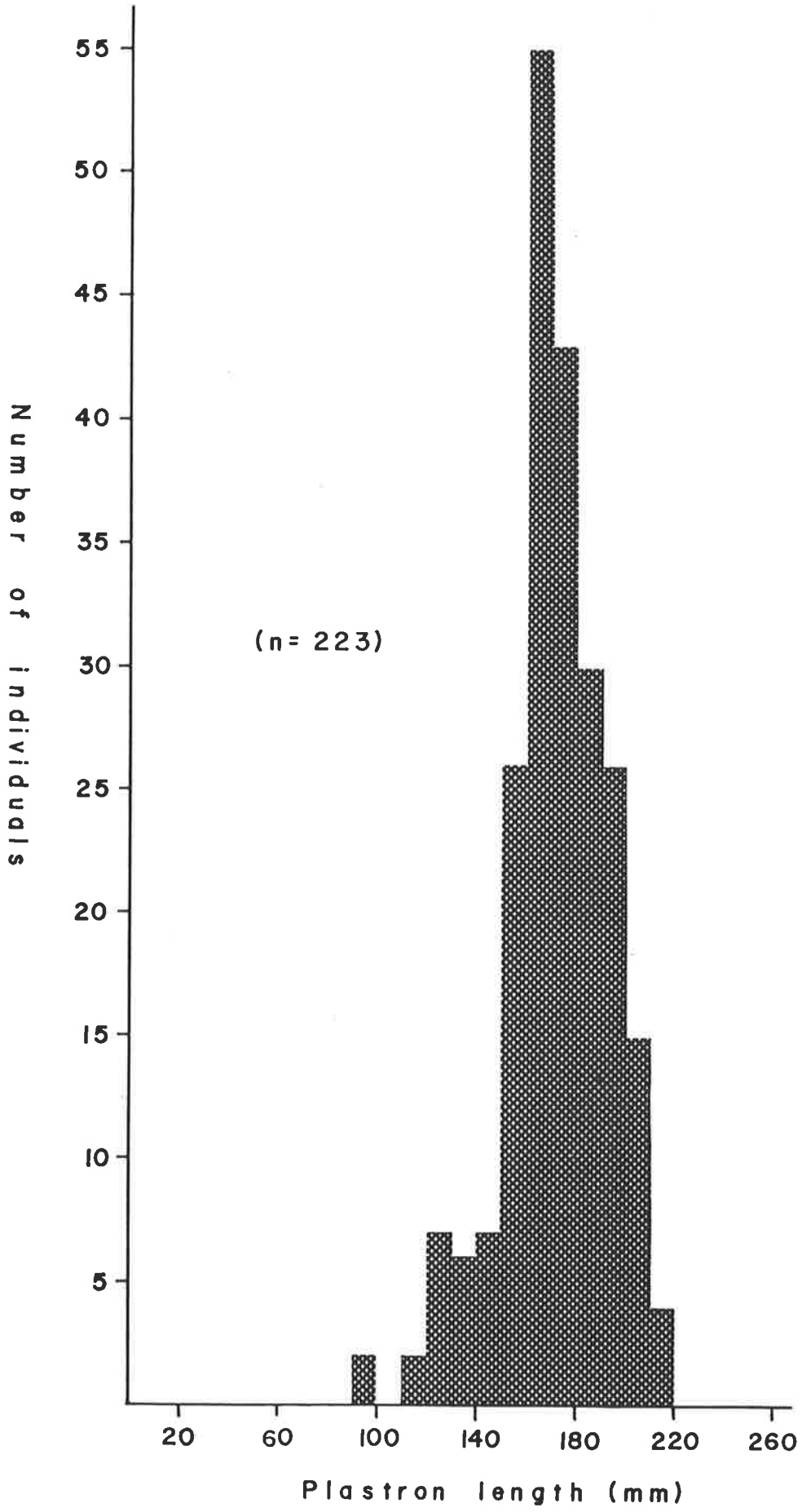


FIGURE 8.3 SIZE CLASSES OF ALL *CHELODINA LONGICOLLIS* COLLECTED IN THE RIVER MURRAY.

have an equal age structure was tested using a G-test (Sokal & Rohlf, 1969). The G-value (9 d.f.) was 27.34 ($p<0.005$) and the null hypothesis rejected.

The significance of the unequal sex ratio is difficult to assess because factors such as sampling biases and difficulties with pinpointing the onset of sexual maturity may have an effect (Gibbons, 1970a).

Sixty-one dead tortoises, many of which had damaged plastrons, were collected along the Cooper Creek. Linear regressions relating plastron length (P) to carapace length (C) and plastron width (W) were calculated from measurements on live specimens;

$$P = 0.82C - 6.95 \quad (r^2 = 0.992, n = 233) \quad \text{and}$$

$$P = 2.86W - 12.11 \quad (r^2 = 0.972, n = 233).$$

The plastron lengths of dead tortoises with damaged plastrons was estimated from these equations and combined with those with complete plastrons (Fig. 8.1).

TABLE 8.2 RESULTS OF COLLECTION METHODS FOR EACH SPECIES

SPECIES	Drum Trap				Hand Capture				Seine Net			
	♂	♀	J	Total	♂	♀	J	Total	♂	♀	J	Total
C. expansa	1			1		2		2				
**C. longicollis*				3				217				1
Emydura sp.	39	134	41	214	1		2	3	7	2	7	16
E. macquarii	58	98	15	171	2	6		8	1	1		2

* In addition 3 were caught on hooks.

8.4 DISCUSSION

8.4.1 Predation

Predation on tortoise nests (n = 369) in the Murray River system of South Australia was found to be 96.5% (including partly destroyed nests). Of the 3.5% of nests left undisturbed by predators, only three (0.8%) completed the incubation unassisted; the other ten were collected or protected by me for other experiments.

Studies on North American species have found high rates of predation on nests. Only 2% of the eggs of *Chrysemys picta* in Michigan survive to hatching (Gibbons, 1968a,b). Five hundred destroyed nests of *Pseudemys scripta troostii* were located at one nesting site but only one intact nest was found (Cagle, 1950). Seventy-one percent of the eggs of *Malaclemys terrapin* were taken by predators in one year and 60% in another year (Burger, 1977) and 94.4% (n = 90) of the eggs of *Chelydra serpentina* were taken in New York in one breeding season (Petokas & Alexander, 1980). Some populations of marine turtles also suffer extensive mammalian predation on the eggs (Stancyk *et al.*, 1980). There is a basic difference between the examples cited and the situation in South Australia. North America abounds with endemic predators, predominantly mammalian, that eat chelonian eggs. Such species include the raccoon (*Procyon lotor*) (Hamilton, 1940; Wilbur, 1975a; Burger, 1977; Wilhoft *et al.*, 1979; Petokas & Alexander, 1980), skunks (*Mephitis mephitis*) (Hamilton, 1940; Cagle, 1950; Wilbur, 1975a), the grey fox (*Urocyon cinereoargenteus*) and perhaps opossums (*Didelphis marsupialis*) (Wilbur, 1975a) and mink (*Mustela vison*) (Petokas & Alexander, 1980). The European red fox (*Vulpes vulpes*) has also been cited as a nest robber in the U.S. (Wilbur, 1975a). In North America snakes take a toll of eggs (Cagle, 1950) but no Australian snake species is known to do so. Gulls and crows also take eggs (Burger, 1977). Presumably the North American chelonians have evolved along with

their predators and have adapted to the pressures of egg predation. In South Australia the main predator, the red fox (*V. vulpes*), is not endemic and may well be placing a great strain on the tortoise populations. Some other studies in Australia have also found high rates of predation on chelid tortoise nests by foxes and other feral animals (Parmenter, 1976; Green, 1980). One study (Vestjens, 1969) found no nest predation in a population near Canberra.

Before the introduction of the fox, there were probably predators not now encountered. Aboriginals were known to consider tortoise eggs a great delicacy (Angas, 1847). Dingoes (*Canis familiaris dingo*) may also have robbed nests. Marsupial predators, such as the Tasmanian devil (*Sarcophilus harrisii*) (Main, 1979; Burbidge, 1981), may have been responsible for predation on eggs. They were all present along both the Murray (Hale & Tindale, 1930; Wakefield, 1964) and Cooper Creek (Stirton *et al.*, 1961). The thylacine (*Thylacinus cynocephalus*) may also have been present (MacIntosh & Mahoney, 1964; Archer, 1974).

Predation on adult tortoises in parts of South Australia may have been high from the Tertiary to Late Pleistocene. Elements of tortoise carapace and plastrons associated with possible crocodile faeces have been found in middle Miocene sediments of the Lake Frome basin (R. Wells pers. comm.); crocodiles are known from Cooper Creek (Stirton *et al.*, 1961). Aboriginals are known to have eaten adult tortoises since they settled the Murray valley (Mulvaney *et al.*, 1964). The present evidence (Fig. 8.1) suggests predation on individuals of all sizes in Cooper Creek, and since essentially the same predators occurred along the Murray previously as now occur on the Cooper Creek, it can be assumed that the same was true on the Murray in the past.

Native cats (*Dasyurus maculatus*, *D. geoffroyi* and *D. viverrinus*) which all occurred along the Murray (Hale & Tindale, 1930; Mulvaney *et al.*, 1964) probably did not take eggs as *D. hallucatus* is not known to take them in Western Australia. There is no evidence to suggest that the present native predators, goannas, ravens and water rats, were more plentiful than they now are.

The above considerations of past predators were made to explore the possibility that egg predation has always been high and that only the predator species have changed over this time. The evidence suggests otherwise. A pair of *E. macquarii* produces an average of 23 eggs per clutch (n = 9) at Lake Bonney and a pair of *Chelodina longicollis* produces 8-24 eggs per clutch (Cann, 1978). High fecundity and a long reproductive life would normally offset high losses of eggs and/or hatchlings. However, the present rate of recruitment of juveniles into the population on the Murray may not be adequate to balance the adult mortality. Slobodkin (1963) divides mortality into four survivorship curves. The North American species fit the type IV curve, where mortality is concentrated at the young stages, as in *Chrysemys picta* where only 2% of eggs hatch but the chance of the hatchlings surviving is very high (Gibbons, 1968a,b). However, a population that is in equilibrium with low nest mortality coupled with high hatchling mortality (Slobodkin Type IV) or moderate mortality at all stages (Slobodkin Type II or III) will decline if egg mortality alone is increased. Throughout such a decline the population will age with the effect being more pronounced in animals with greater longevity, such as tortoises. This reflects the situation under study. Although Figure 8.1 indicates that there is a higher predation rate on sub-adult tortoises in the Cooper Creek, more importantly it indicates that there is significant predation on all size classes. This is contrary to the suggestion that the rate of predation on hatchlings

is inversely proportional to their size (Wilbur, 1975b). If this sample (Fig. 8.1) is biased against very small animals, the difference between this and North American populations is even greater.

Other lines of evidence can be used to infer a low rate of egg predation in the past. Some North American studies (Christiansen & Moll, 1973; Wilbur, 1975a,b) have suggested that a strategy of multiple egg clutches in a season is a response to reduce egg predation. *E. macquarii* has a relatively low incidence of multiple clutches (<10%, n = 85) (Thompson, unpublished data) and in some areas where the nest predation rate is very low *Chelodina longicollis* has only one clutch per year (Vestjens, 1969). This may indicate that egg predation has been low in the past. Species occurring in Australia that have evolved where mammalian predation is high, such as marine turtles, still have a high incidence of multiple clutches (Bustard, 1972). The incidence of multiple clutches in *C. longicollis* and the *Emydura* in the Cooper Creek is unknown.

8.4.2 Population Age Structure

To estimate the probable age structure of the *E. macquarii* population in the Murray before the introduction of foxes, a study of the Cooper Creek *Emydura* species was undertaken. This is the only area in South Australia where tortoises occur in the virtual absence of foxes. Dingoes are known to eat foxes (P. Aitken pers. comm.) and this may be the cause of the scarcity of foxes. Foxes are also absent from areas of Western Australia where dingoes are common (D. King pers. comm.). I saw no evidence of foxes in the Cooper Creek area and residents of the area confirmed that foxes are rare. However, dingoes are common. Dingoes occurred along the Murray before foxes. Other nest predators occurring along the Murray, water rats, ravens and goannas, also occur along the

Cooper. The age structure of the two *Emydura* populations was shown to be very different. There were very many more juveniles in Cooper Creek than in the Murray (Fig. 8.2). As both *Emydura* populations were collected in the same way (Table 8.2) any biases resulting from capture techniques can be ignored.

The results can be interpreted in the following way. On the Murray a very high percentage of eggs are destroyed and there is little juvenile recruitment into the population. This situation is reflected now in the population of large (= old) animals. However, on the Cooper Creek, egg predation is low and large numbers of juveniles enter the population. Hence the population has large numbers of individuals of all size classes. The apparent lack of individuals of the Cooper Creek *Emydura* sp. smaller than 110mm plastron length (Fig. 8.2) is an artifact of the capture technique caused because specimens smaller than this could escape from most of the traps.

It is difficult to relate the data on the age structure of the tortoise populations to the historical distribution and abundance of foxes because the longevity of the tortoises in the field is unknown and the spread of foxes is undocumented. The scant information on the history of the fox in Australia was collated by Rolls (1969). After a successful introduction to Victoria in the 1870's they were first recorded on the Murray in Victoria by 1886. As a result of other apparently unrecorded releases, foxes were seen on the Coorong in South Australia in 1888. The population grew quickly and presumably peaked early. In 1952 there was a sharp reduction in the numbers of foxes throughout Australia with very little successful breeding for 2-3 years due to an epedemic of mange. This should have allowed a large recruitment of juvenile tortoises at this time and many of the large specimens present in the population now

(Figs. 8.2, 8.3) may have been recruited then.

CONCLUSION

The presence of many old *E. macquarii* in the Murray indicates that the population is presently undergoing a change as a result of nest predation by foxes.

There is some juvenile recruitment into the population but this is likely to diminish as these old individuals die. The population size is likely to stabilise at a very much lower level. As the number of nests decreases, the rate of predation may also decrease, leaving a greater percentage of nests to hatch, as was shown in a population of *Chrysemys picta* that declined over 20 years (Wilbur, 1975a).

These conclusions can also be applied to *C. longicollis*. This species was found, by dissection, to reach sexual maturity at 140-150mm plastron length. Therefore it also is made up of many old individuals with low juvenile recruitment (Fig. 8.3) and is likely to decline.

9

IMPLICATIONS FOR MANAGEMENT

The physiological and ecological studies of the eggs of *E. macquarii* have implications for the conservation management of the species and possibly for other species of Australian Chelidae. The only Australian chelid to be studied in detail from a population management point of view is the rare western swamp tortoise *Pseudemydura umbrina*, which has peculiar and unique problems (Burbidge, 1967, 1981). However, increasing agricultural and recreational uses of the limited Australian water resources in general, and of the River Murray in particular, is likely to result in the need for careful management to ensure the maintenance of the present abundance and diversity of wildlife. This study may provide useful information to ensure the maintenance of viable populations of *E. macquarii*.

The answers to several important management-related questions can be gleaned from this study. Such questions include: What sort of areas need to be set aside for successful nesting?; What sort of protection from predators should be provided?; Is the movement of eggs to safer places for incubation likely to be detrimental to their development?

Nesting Areas. *E. macquarii* does not select nesting sites by the characteristics of the soil (Chapter 6). They appear to emerge adjacent to their position when environmental cues initiate nesting behaviour. Nests are only abandoned before oviposition if an obstacle prevents proper nest construction. Therefore any area in which tortoises now nest can be regarded as a candidate for intensive conservation efforts. Tortoises may avoid areas subjected to flooding. However, regulation of the River Murray has now eliminated small floods. Also the nesting period is usually after peak flow, when the water is receding in times of flood.

Predation. Predation by the introduced red fox appears to be having a large effect on the rate of recruitment of young *E. macquarii* into the population. Therefore some method of protection from foxes seems necessary to prevent a decline in populations of *E. macquarii*. The obvious answer is to eradicate foxes from nesting areas, but this is very difficult. All of the nesting sites that I identified along the Murray are near to human habitation, both rural and urban, which makes shooting foxes hazardous and ineffective. The nesting areas could be baited with sodium fluoroacetate (1080) or strychnine laced baits prior to the nesting season as is done for *Pseudemydura umbrina* (D. King, pers. comm.). However, this type of control is nonspecific and, as the nesting areas are near to human habitation, many local dogs are likely to fall victim to the baits. An alternative is to fence off nesting areas. A fox proof fence, that would allow endemic predators access to the nesting areas, could be constructed. To be fully effective it should be two metres high, buried in the ground, have an overhang of barbed wire and extend a considerable distance into the water (S.A. Vertebrate Pest Control Authority, pers. comm.). Such fences are expensive to erect, unsightly and require a fair amount of maintenance. Alternatively a fence, about a metre high, consisting of electrified wires every 30 cm with a non-electrified wire in between, and constructed at an angle of about 75^{o} to the ground leaning away from the nesting site may be an adequate deterrent to foxes and significantly reduce egg losses (S.A. Vertebrate Pest Control Authority, pers. comm.). However this remains to be tested.

An alternative to protecting eggs *in situ* is to remove a percentage to an area where predation is controlled or even to artificially incubate eggs. Eggs collected in the field and incubated in suitable conditions experienced hatching rates up to 100%, so movement does not damage eggs. Eggs could be collected and reburied in artificial nests constructed to

resemble real nests so that the conditions of incubation reflect the natural nest environment. Any soil type could be used for construction of nests, provided there is no possibility of the eggs being inundated by water. The position of the artificial nest in relation to sun and shade is not important because sex is determined independently of temperature in *E. macquarii*. As the eggs of *E. macquarii* have the ability to synchronise hatching, it would not matter if eggs from clutches of slightly different ages, but from the same location, were mixed in artificial nests. Hatchlings from artificial incubation should be returned to the water adjacent to the site of original collection to maintain the integrity of the population.

The implimentation of conservation measures at the nesting stage of the life of *E. macquarii* assumes a knowledge of the structure of the whole population. This knowledge is lacking. My results indicate that the population in the Murray is declining as a result of intense predation by foxes. Further monitoring of the population is required to confirm this suggestion. It is important to act on this information while the population is still large and apparently healthy rather than to wait until remedial action is necessary, as in the case of many rare species (e.g. *P. umbrina*, Burbidge, 1981; Galapagos tortoises, MacFarland & Reeder, 1975).

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