ASPECTS OF THE OSMOTIC AND WATER BALANCE OF THE NEW ZEALAND NATIVE FROG *LEIOPELMA HOCHSTETTERI* FITZINGER, AND THE AUSTRALIAN WHISTLING FROG *LITORIA EWINGI* DUMERIL AND BIBRON.

A thesis presented in
fulfilment of the requirements for the degree
of Master of Science in Zoology
at Massey University

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Le. hochstetteri. Location I (Tokatea Ridge), Coromandel Peninsula, indicating external morphology and similarity to rock colour. Note ridged appearance of skin.

Li. ewingi. Foxton Beach, indicating external morphology and colour pattern. Note smooth appearance of skin and digital pads.

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ABSTRACT

Rates of dehydration and rates of water uptake when hydrated or dehydrated are described for two species of frogs of similar size from different habitats. No detectable differences in rates of water loss in frogs of both species of comparable size were noted. Considerable differences were seen in rates of water uptake. These uptake rates were lower in hydrated and dehydrated Le. hochstetteri than in hydrated Li. ewingi. Differences in rates of water uptake were reflected in measurements of skin permeability and blood plasma osmolality. Rates of water uptake in Li. ewingi were dramatically increased after dehydration, and it was proposed that this was due to hormonal mediation. The osmotic permeability of different skin regions in frogs of different species may vary in the presence or absence of oxytocin or vasopressin. This was not observed in Le. hochstetteri where the skin exhibited relatively uniform permeability, but was seen in Li. ewingi and Li. aurea. In these two species, the abdominal skin was more permeable and more readily stimulated by oxytocin or vasopressin than the dorsal skin. Oxytocin and vasopressin also increased the short circuit current (inward Na^+ transport) through both dorsal and ventral skin in Le. hochstetteri, but most noticeably through the ventral skin in Li. ewingi and Li. aurea. The skin was observed to be thinner in Li. ewingi than in Le. hochstetteri or Li. aurea. Thin areas in the ventral pelvic integument of Li. ewingi and Li. aurea and the presence of epidermal capillaries in these two species are thought to be of importance in water uptake. It has been suggested that water uptake mechanisms are a major factor determining the distribution of the three frog species.

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1. GENERAL INTRODUCTION.

Amphibia are generally regarded as water-loving animals with slimy skins. However, this generalization does not recognise the fact that, in common with many animals, the amphibia have come to occupy diverse habitats during their evolution.

The Amphibia are thought to have arisen from Crossopterygian fish (Villee et al. 1968, Bentley 1971) which were successful in the fresh waters of the Devonian but have dwindled to a few relict species today. In their turn, the Amphibia have given rise to the reptiles. These tetrapod vertebrates have improved on the terrestrial attributes of their amphibian ancestors, and, in having evolved means of conserving body water, in reproducing on land and, to some degree, controlling their body temperature, the reptiles have overcome the essential problem of terrestrial life.

The contemporary amphibia are represented by three orders. The Apoda or caecilians (of which there are about 17 genera) are legless, burrowing wormlike forms confined to the tropics. The Urodela or caudata (43 genera), contain the newts and salamanders. They retain the primitive body form with short legs, long trunk, well developed tail, and are mostly confined to temperate region in the northern hemisphere. The Anura, or frogs and toads, are the most numerous of the three orders, containing more than 200 genera which are widely distributed around the world, where they occupy habitats ranging from freshwater ponds and streams, to arid deserts, and in one species at least, to sea-water.

The amphibia exhibit a number of physiological differences from their ancestors and descendants, but probably the major one that affects amphibian osmoregulation is the adoption by most adult forms, of atmospheric instead of aquatic respiration. The amphibia utilize

their skins for gaseous exchange and, as a result, the skin is also more permeable to water than both the Crossopterygii with their scaly skin, and the Reptilia with their dry and horny covering.[1]

A second major factor influencing osmotic exchanges in amphibians is the need to lay their eggs in water, the aquatic larval form later metamorphosing into an amphibian adult.

Because of their diverse habitats and their evolutionary position, amphibia are popular experimental animals. Many workers have attempted to relate certain physiological and adaptive properties of them to these habitats. Some definite correlations do exist, with skin permeability, blood osmoconcentration, rates of water uptake by hydrated animals and after dehydration, the ability to survive prolonged hydration or dehydration; the adaptive value of these properties to their possessors is clear.

The reverse should also be possible. Knowing some details of amphibia from different habitats, one should be able to measure some of the properties described above and explain why a certain frog is found living where it is, or whether the habitat where found, does not in fact describe the entire range of conditions under which the animal may be found. Is it not possible also, that a primitive amphibian, thought to be restricted

[1] Recent data presented by Licht and Bennett (1972) casts doubts upon the assertion that scales are an adaption in reptiles to retard water loss. A gopher snake Pituophis melanoleucus catenifer lacking dorsal and lateral body scales was used to evaluate the physiological importance of reptilian scales. In tests of pulmocutaneous water loss and heat transfer, no difference was observed between the scaleless animal and a normal individual of comparable age and size. Licht and Bennett concluded that the comparatively low rates of evaporative water loss characteristic of reptiles relative to other terrestrial vertebrates must be viewed as a function of some aspect of the integument other than scales per se.

to a particular area by locomotory deficiences, could also be deficient in some physiological systems refined by more terrestrial counterparts? Or that, contrary[2] to generally accepted surface area to volume relationships, a small frog can live in drier areas than a larger frog because it possesses more efficient water conservation mechanisms? These are thought to be definite possibilities. This study was therefore undertaken to discover some of the important characteristics of two species of frogs that enable them to live where they do, and which limit them to these areas.

The indigenous frogs of New Zealand are restricted to three species of the family Leiopelmidae[3] genus Leiopelma: Leiopelma hochstetteri Fitzinger, Leiopelma archeyi Turbott and Leiopelma hamiltoni McCulloch, all of small and similar size.

Work on the New Zealand native frogs has been primarily restricted to anatomical, developmental and habitat studies (Archey [4] 1922, Turbott 1949, Szarski 1951, Stephenson E.M. 1951, 1952, 1955, 1960, 1961,

[2] On the assumption that a small frog, with greater surface area to volume ratio will lose proportionately more water from its body surface per time than a large one. It may be thought then that natural selection would have favoured larger forms in drier areas, although this ignores the advantages of small forms in finding suitable microhabitats.

[3] Correctness of this familial grouping is discussed by Stephenson E.M. & N.G. (1956), and Stephenson, E.M. (1961), who suggest the separation of Noble's suborder Amphicoela into two families, Leiopelmatidae and Ascaphidae, and by Robb (1973) who states that the family name Ascaphidae has priority, but that the Leiopelmidae is most widely used for the group.

[4] Archey's paper described L. archeyi Turbott, not L. hochstetteri Fitzinger (Turbott 1949, Stephenson N.G. 1955).

Stephenson N.G. and Thomas E.M. 1945, Stephenson E.M. & N.G. 1957 Stephenson, N.G. 1951 a & b, 1955, Crook *et al*. 1971). The anatomical and developmental studies have established them, together with *Ascaphus truei* Stejneger of North America (an aquatic species, inhabiting cold mountain streams (Noble 1954) as the most primitive living frogs. Stephenson (1951 b) indicates the close relationship between *Leiopelma*, *Ascaphus* and the Urodeles, in details of adult anatomy and development.

Habitat studies have reported the nocturnal New Zealand frogs as living in higher altitude areas and more inaccessible regions; areas of high annual rainfall, and generally, high humidity.

Leiopelma hochstetteri and *Leiopelma archeyi* are mainly known from the Coromandel Peninsula, where they exist together (Stephenson E.M. & N.G. 1956 ; and my own findings.) though *L.archeyi* is also known to exist in drier areas than those inhabited by *L.hochstetteri*. *L.hochstetteri* is also known from Warkworth, the Waitakeri Ranges and coastal areas south of Coromandel Peninsula (Stephenson E.M. 1961).

General features of the Coromandel habitat include rain and mist in the high ridges, generally rocky country with scattered small rocky streams, and vegetation ranging from sparse and open on the actual ridge tops, to bush forest with many ferns and moisture-loving mosses on the more sheltered slopes.

Leiopelma hamiltoni is known from restricted areas on Stephens and Maud Islands in Cook Strait. On Stephens Island, *L.hamiltoni* lives in the formerly forested but now exposed "frog bank" which may have been formed by ridge slumping, creating a deep pocket of soil and rock (Crook *et al*. 1971). No free surface water exists. The more favourable forest habitat on Maud Island does not have permanent streams, though the gullies in which the frogs are found, under boulders, logs, and

rock-accumulations, do carry water after heavy rain (Crook et al.1971).

Griffiths (1963) regards the geographic range of the family Ascaphidae (Ascaphus and Leiopelma) as relict, following from the emergence of non ascaphid forms. He suggests that the failure of the Ascaphids to compete with these forms is due to locomotory disadvantages inherent in their primitive anatomy. "The survival of recent Ascaphid genera and the retention by them of a common generalized morphology almost certainly reflects their isolation in rigid habitats" (Ritland 1955).

Three introduced species of frogs of the family Hylidae [5] also exist in New Zealand, having been introduced from Australia: Litoria aurea Lesson, the Green or Golden Bell frog, Litoria ewingi Dumeril and Bibron, the Brown Tree frog or Whistling frog, and Litoria caerulea Boulenger, the Great Green Tree frog. The Hylidae (including the true tree frogs) may be arboreal, terrestrial or aquatic, and some are fossorial (McCann 1961).

L. aurea is a true amphibian, usually found in or near streams, lakes, or pools, though in damp and dark conditions it may wander some distance from permanent bodies of water (McCann 1961). During the day, this species can often be found floating in water, (refer to plate 3) basking in the sun and,at night, is easily caught from water. L. aurea appears to have a New Zealand-wide distribution where suitable conditions exist.

L. ewingi has been variously described as predominantly terrestrial (Elkan 1968), remaining close to water throughout its life (Warburg 1964), occupying bush-clad country of swampy nature where the humidity and

5 After Tyler (1971) and following Watson et al. 1971, the Australian species previously included in Hyla are now placed in the genus Litoria Tschudi 1838.

herbage afford it a suitable environment (McCann 1961), and as a nocturnal, arboreal species often ascending several feet above the ground. (Marriner 1907). L. ewingi is common on the West Coast of the South Island and in Southland. After being successfully introduced into the North Island in 1948 (on the West Coast near Himitangi) (McCann 1961, Gill 1973), the species has spread considerably from this liberation point (Gill 1973).

L. caerulea, like L. ewingi, is nocturnal, resting during the day in logs or on foliage. Little is known of the distribution of L. caerulea, though McCann (1961) states that it is present in parts of the North Island but has rarely been found.

Water is a requirement for the larval development of all three introduced speciea, the adults mating in water and the fertilized eggs producing free swinging tadpoles. The three native species are unusual in that intracapsular larval development occurs, the tadpole developing inside the egg in generally damp situations and hatching out as a fully formed froglet. However, this mode of development may not be an adaptation to terrestrial living or dry conditions; rather it may have been made possible by the generally wet conditions prevailing (Archey, 1922, Stephenson, N.G. 1955). The important experiment by Archey (1922), confirmed by Stephenson, N.G. (1955) showed that in fact, young tadpoles liberated from their eggs during development continued normally in water. This intracapsular mode of development may not be as specialized as first thought.

The present study involves Leiopelma hochstetteri taken from Tokatea ridge and two other locations on Coromandel Peninsula (refer to figures 1 and 2). This species is believed to be the most aquatic of the three New Zealand natives, being commonly found in or near streams (Turbott 1949) and possessing half-webbed toes [6]

[6] L. archeyi has only slight development of webbing (usually less than one quarter), while in L. hamiltoni, webbing is absent.

(Stephenson, E.M. 1961). However, this frog has since been found on a number of occasions well away from surface water (Stephenson, N.G. and Thomas, E.M. 1945), a finding confirmed by the present investigation.

It was of interest therefore, to compare L. hochstetteri with L. swingi, of similiar size and weight (refer to Appendix I). as terrestrial animals. It was hoped to gain some idea of their relative responses and abilities in water conservation, and to see whether these frogs, so different anatomically and developmentally, were in fact well equipped for land living. A more careful and detailed description of the habitats of these two species of frogs is therefore relevant.

2. Habitat Studies and Description of Animals.

Twenty specimens of Leiopelma hochstetteri were collected from the Coromandel area at three sites in late April (refer to figure 2).

Collection Site I was Tokatea Ridge (altitude 396-427 metres) where the road from Coromandel on the West Coast to Kennedy Bay on the East Coast of the peninsula crosses the range. A track leads along the eastern side of the ridge below the summit and collection was made approximately 1.6 km north along this track. The wet and muddy conditions made the use of gumboots necessary. Conditions were generally very unpleasant on the saddle with cloud and mist, and the wind gusting up to 9.8 m/s (22 m.p.h.). Several metres along the track, in the shelter of the hill, the wind velocity had dropped to 4.5 m/s (10 m.p.h.), and this further reduced to a negligible value at the collection site where thick bush prevailed. This illustrates the generally sheltered nature of the habitat. Though it was not actually raining, mist and absence of wind made the humidity very high (95% relative humidity at 16°C). (Wet-and-dry bulb thermometer). No permanent streams or ponds were seen

FIGURE 1.

North Island, New Zealand.

Collection sites of Le. hochstetteri and Li. ewingi ■

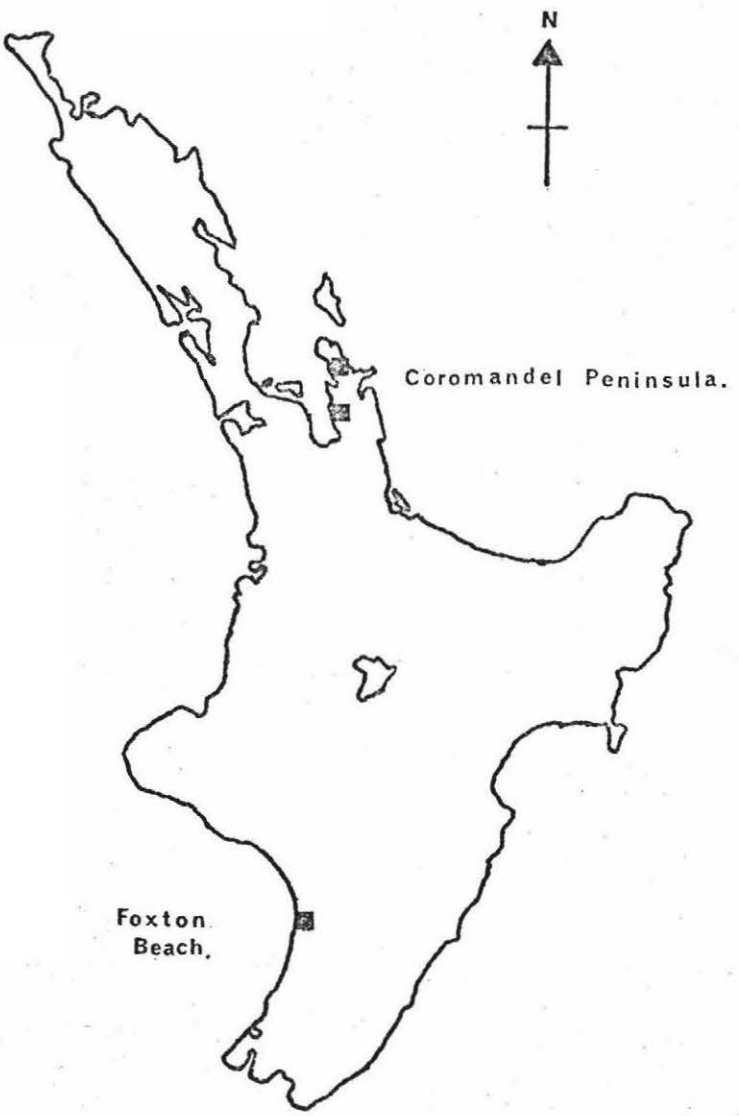


FIGURE 2.

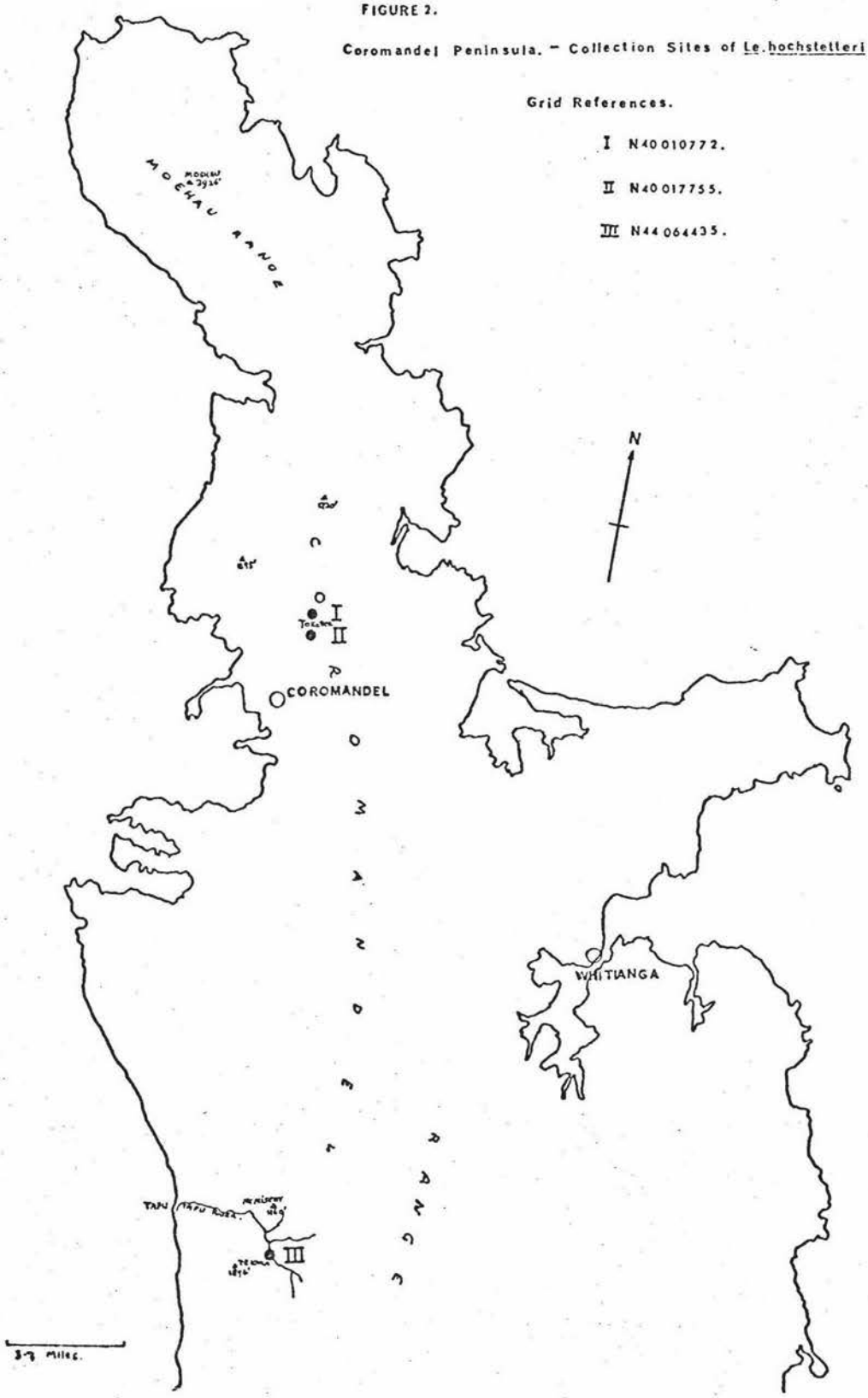
Coromandel Peninsula. – Collection Sites of Le. hochstetteri ●

Grid References.

I N40010772.

II N40017755.

III N44064435.



in this ares, the frogs mainly being found comparatively easily in damp rotting vegetation, amid piles of rock alongside the track (refer to plate 1.) and, on several occasions, in apparently dry situations under rocks on the track bank. However, it must be noted that the high ambient humidity would reduce water loss by evaporation to a minimum, thus allowing the frogs some independence of surface water. Similar numbers of L. archeyi were also found, particularly in the damp rock piles along with L. hochstetteri.

Collection Site II was in a rocky stream on the western face of the range below the Saddle (altitude 366 metres), where it was also sheltered, though little mist was present. Conditions were generally cool and shady (94% R.H. at 15.5°C) and frogs were found amongst the rocks and woody debris present, along the banks, and out of the main body of the water. There, as in Location I, the jumbled piles of rock provided many cracks and crevices into which the frogs could wriggle. L. hochstetteri was the only species found here, and at obviously greater density than in Location I.

In all three locations, the frogs initially sat quietly and, being well camouflaged (refer to frontispiece) were very difficult to see in the shaded surroundings.[7] They often leapt very rapidly to new cover, disclosing their presence. Once in water, with vigorous kicking of alternate hind legs, they moved with the current, sliding over rocks and pebbles before finding refuge under a new boulder or in the cracks and crevices amid a pile of rock.

[7] Robb (1973 discusses more recent findings of L. hochstetteri south eastward through the Bay of Plenty, Opotiki, the Waioeka Gorge, Matawai, East Cape, and from the Hunua Range south of Auckland. She suggests that because of the very good camouflage of the native frogs and, as they are very difficult to find, the full extent of their distribution has not been realized. "It is becoming more and more apparent that the frogs are highly inconspicuous rather than excessively rare" (Robb 1973).

Plate 1.

Moist rock pile habitat of *Le. hochstetteri*. Collection Site I, Coromandel Peninsula.

Location III (to the south of locations I and II) and at lower altitude (274 metres) consisted of another, slower stream, less rocky in nature and with more soil between the rocks and on the stream bed. The frogs here were less numerous than in the other two locations, possibly because the soil had filled up many of the crevices between rocks.

The presence of higher numbers of the frogs in the wetter areas and streams suggests that these frogs are predominantly aquatic. Indeed, on revisiting areas I and II in late November, considerable differences were noted. Conditions were more rigorous; there was a slight breeze and the sun was shining. The track was now completely dry and hard, the rock piles dry and the humidity considerably lower. Native frogs were searched for in the area where they were previously collected, but none was found. Ground cover in the bush still appeared damp, but the overall impression was one of dryness. However, Location II (rocky stream) yielded a frog after a few moments searching, which supported the idea that L. hochstetteri exists in areas of high moisture. In fact, it has been stressed repeatedly (Archey 1922, Turbott 1949, Crook et al. 1971, among others) that where surface water does not exist, the native species are only found where conditions are damp and where rain and mist are prevalent.

The 20 specimens of L. hochstetteri collected ranged considerably in length from 20.5 to 39.7 mm and in weight from 0.99 to 6.26 g. Colour appeared to remain constant in pattern and intensity in individuals. Dorsal colouration ranged from dark brown-black through golden and rusty brown to dark olive green[8] and light green.

[8] Stephenson E.M. (1961) reported the finding of a bright green specimen of L.hochstetteri on Tokatea ridge and stated that no other instance of green colour in this species had been reported. The two green specimens caught in this study (one of them my smallest frog) both appeared to be true L.hochstetteri species. Similarly coloured L.hochstetteri have also been noted by another worker (B.D. Bell pers. comm.).

The ventral surface was invariably lighter in colour, ranging from light grey to dirty cream. All specimens had generally well defined forearm and leg banding in brown and gold, and partial toe webbing. The sex of these frogs could not be distinguished externally except where, in several specimens, large white to cream eggs c could be seen through the ventral body wall.

The specimens of L. ewingi were collected from a solitary flax bush [9] at Foxton beach (refer to figures 1 and 3). The flax bush (refer to plate 2) was situated in a grassed reserve that was regularly mown, and which also contained a large pond approximately 25.5 metres away. Frogs could be found sitting near the base of the leaves when these were parted, where it was cool and where evaporation would be reduced. They were also found in grass and other plant material at the base of the bush, and occasionally, further up the leaves.

Frogs were collected from this bush at intervals during the year; reproductively active males and females were invariably found, though none was seen in the pond during these infrequent trips. The weather was generally cool, with little wind during the collections; this may be thought to have favoured the capture of these frogs. However, on revisiting the area after the completion of experiments towards the end of January, the frogs were still present in good numbers. The day was very hot with a gentle breeze blowing, rustling the flax leaves. The grass ridge between the bush and the pond now consisted of a strip of bare dry sand 3-4.6 metres wide, while the remaining area consisted of sand interspersed with stunted grass. The flax bush was only half its former size reflecting the general dryness of the area and the

[9] Mr. A. Gates of Orua Downs (on whose property the whistling frog described by Barwick (1961) was caught) informed me of a group of L. ewingi in a woodpile on his farm, but a search for them proved unsuccessful.

FIGURE 3.

Collection sites of Li. ewingi ● and Li. aurea ■.

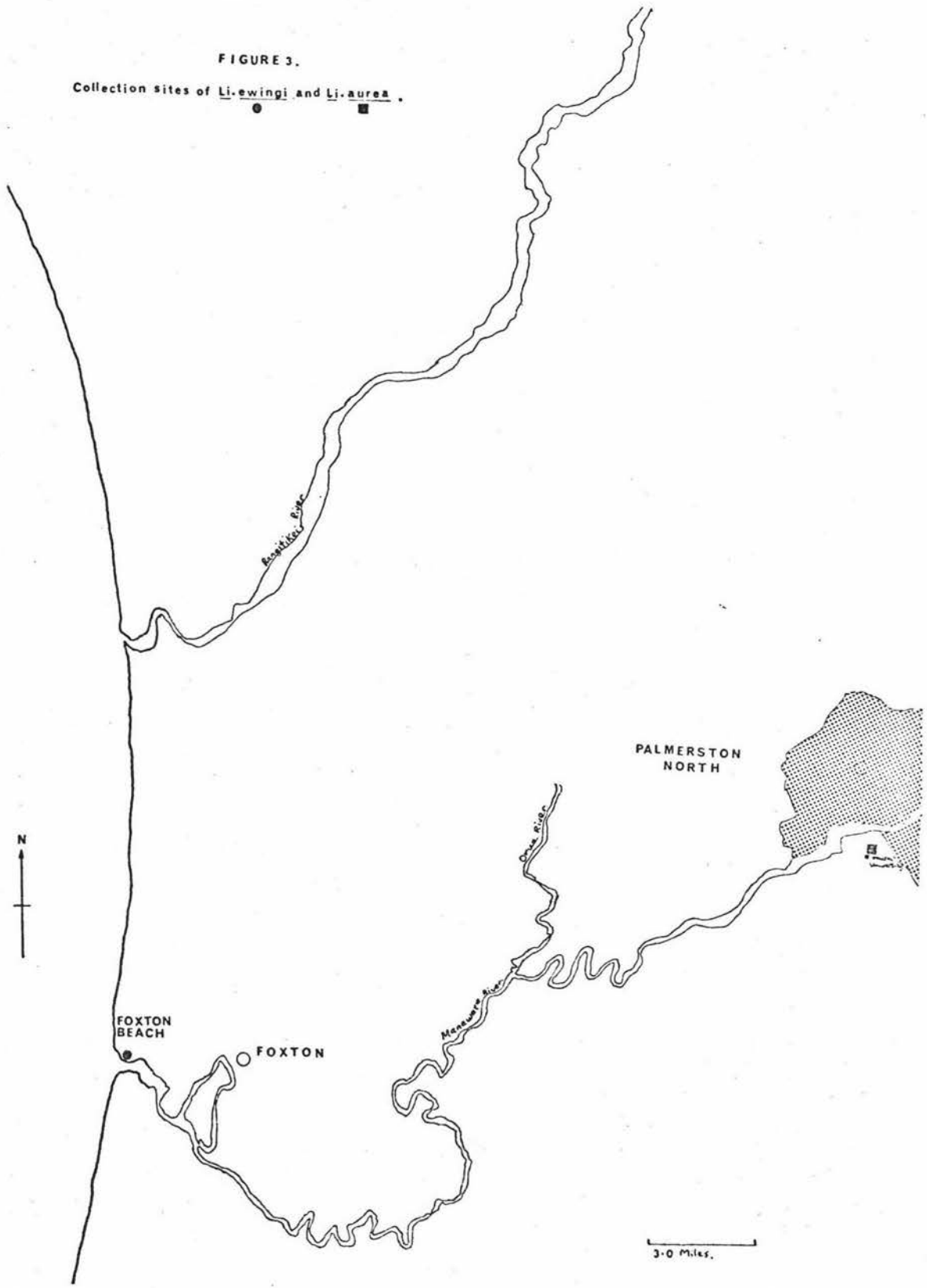


Plate 2.

Flax bush at Foxton Beach. Collection Site of Li. ewingi. A belt of macrocarpa trees is evident behind the flax bush. The edge of the pond can be seen on the extreme right of the plate. The ridge, grassed in winter but exposing dry sand in summer, is seen in the centre of the plate.

Note. The sign refers to a water tap for campers.

vegetation round the base of the bush was much drier though the sand under the dead leaves was still damp. Frogs were readily found between the flax leaves, and some were also further up, exposed to the breeze rocking the leaves.

Recently caught L. ewingi females often laid eggs, not only in water, but on leaves, sand, and rocks. In some cases these proved fertile, and a clump was retained to follow development. The young tadpoles soon came to lie on their backs and created currents, sweeping fish food towards them. As development proceeded, with first legs and then arms appearing, they would crawl up the sides of their containing jars or on to the rocks provided out of water. In fact, their climbing abilities were underestimated and all except one young froglet were lost in this way. The developing frogs were all at a similar stage (tail just starting to be resorbed) and, with no lids on their containers, they were able to climb up the walls and escape. The one remaining froglet (length 13.4 mm) would invariably sit right up under the jar lid, and when fully developed, was kept for some weeks. During this time, it was never observed in water, whenever examined at various times during night and day. L. ewingi therefore appears to be fairly independent of water from an early age. This observation may well tie in with in vitro experiments carried out by Warburg (1971) on tadpoles of Pelobates syriacus. At various stages of development including the stage when the hind limbs were well developed, no effect of oxytocin was observed. However, soon after emergence on land, when a stump of the tail was still present, the froglets started to respond to this hormone which promotes water uptake and retention.

Males of this species are easily distinguished from females by secondary sexual characteristics and by size, the females being longer and heavier (30.5 to 38.8 mm, 3.31 to 6.38 g) than the males (30.2 to 32.1 mm, 2.21 to 2.91 g). Non-pregnant females could be

recognised by the absence of black thumb pads.

All specimens had a wide brown mid-dorsal stripe with a variable border of black dots. The sides of the body were generally lightish brown to mushroom in colour and the granulate ventral surface white to cream. Considerable colour variation was noted though the dorsal skin of individual frogs in differing light conditions ranged from dark chocolate brown through to very light cream. Constant colour was shown by the ventral side of the hind legs which were orange with black spots. All these frogs possessed good toe webbing and sucker-like digital pads.[10]

Specimens of L. aurea were also used in some of the studies. They were collected from a pond near Massey University (refer to plate 3) at night with the aid of a torch and a net. These frogs were considerably larger and heavier than the other two species used (18.3 to 24.9g), and were generally light to dark green on the dorsal surface with scattered gold colourings, while the granulate ventral surface was yellow-cream to white. The ventral side of the hind legs was often bluish-purple in colour.

3. HOUSING AND FEEDING

All frogs used were kept at Massey University in a room with concrete floor, part of which was continually wet. A small grille was the only permanent opening to the outside. Temperature fluctuated in this room between 7-9°C in winter, and 18-19°C during the summer. The

[10] These are not suction devices, but elaborate friction and adhesion mechanisms, the epidermal cells projecting like short bristles against a surface with a complex series of tubular glands pouring their adhesive secretion on to the surface of the pad (Noble 1954).

Plate 3

Pond near Massey University.
Collection Site of *Li.aurea*.
Plate shows frog (arrowed)
floating in water during the day.

cold concrete floor acted as a heat sink, limiting sudden temperature fluctuations, and conditions were shaded and cool.

All frogs were kept in containers of varying description. The native frogs were kept in their groupings corresponding to collection sites. The eight frogs from Location I were housed in a large aquarium, the floor of which was covered with damp soil, damp sphagnun moss, rocks and rock fragments and several large pieces of wood and bark for shelter. More by accident than design, there was no permanent water in this aquarium, as it leaked badly. Water was therefore replenished every few days by which time it had often disappeared, leaving only wet rocks. However, the use of glass lids presumably maintained humidity at a high level.

The remaining twelve native frogs were housed in a combination of smaller aquaria, and round plastic food containers with a series of fine holes in their lids. These generally contained a small pool of water surrounded by mud and large damp stones. Further back from the pool, large rotting leaves and pieces of wood and rock rested on the earth and rock fragment floor. Where plastic food containers were used, the habitat consisted of a jumbled pile of rocks placed in water. The frogs could be found sheltering in the dark and shade during the day in crevices between rocks or under the rotting vegetation. They were rarely seen in the actual water pools. At night, though not equipped with digital pads for climbing like the whistling frogs, they would commonly be found sitting up the walls of their respective containers (up to 35-40 cm above the ground) though they would rapidly return to shelter and shade when the lights were turned on.

The whistling frogs (L. ewingi) were initially kept under similarly very damp and humid conditions, and some losses were experienced. Drier conditions in the

cages were then tried (dry aerial foliage and a container of water among some grass) and glass lids were partially replaced by plastic mesh to lower the humidity. These conditions appeared to suit the frogs better. They were often found sheltering under flax leaves provided, during the day, though some could be found aerially in twigs and branches, or up the sides of their aquaria.

Specimens of L.aurea were kept in two sorts of containers. The first consisted of two plastic boxes, one fitting into the top of the other. The bottom box had an inlet and outlet so that fresh water could be constantly circulated. A section of the floor of the top container was cut on three sides and bent down to form a ramp into the lower water section. The upper (terrestrial) level contained pieces of clay flower pots as refuges and a fine muslin lid was provided. Most of the frogs remained in the water though several could be found resting in the top compartment at most times. At feeding time all the frogs would eagerly crawl up the ramp and catch the flies as they came to rest on the walls or roof of the container.

The second method of keeping these green frogs was in clear plastic lunch boxes with holes in the lids and bases, and containing some sphagnum moss. These boxes were stacked on each other with water dripping down through the column maintaining the frogs in a moist condition. [11]

All cages and aquaria were inspected for dead flies and droppings every few days, and the water was changed periodically.

Initially the frogs were fed on a mixed diet of fruit flies (Drosophila- vestigial wing strain), blowflies and houseflies, occasionally supplemented with

[11] Considerable numbers of L.aurea have been successfully kept in this way for many months by the Department of Physiology and the Department of Zoology at Massey University.

moths caught in a light trap. The Drosophila were narcotized with CO_2 and shaken into the frog cages. Blowflies and houseflies were placed, in their rearing cages, in a coolroom and removed when lying chilled on the cage floor. They were then shaken directly into the frog aquaria or if they had revived sufficiently to fly, were lightly etherized. When the flies began to stir, they were eagerly seized and devoured.

However, owing to the time spent in rearing these different insects, the frogs were later mainly fed houseflies. They thrived on this diet and remained in good health.

The experiments carried out, fall quite naturally into two categories; (a) whole animal experiments and (b) those involving some of the isolated systems of the animal. These systems, operating together, may partially or completely explain the varied reactions of a frog in differing situations.

It was decided to measure rates of dehydration and water uptake in the two species of similar size and weight (Le.[12] hochstetteri and Li.ewingi) and to include the larger Li.aurea in measurements of blood osmolality, skin permeability and in studies of the structure of the skin.

[12] Le. will be used to denote Leiopelma and Li. to denote Litoria, to avoid confusion.

4. WHOLE ANIMAL EXPERIMENTS

(a) Dehydration Studies

i Introduction.

The ability to prevent excessive water loss from the skin by evaporation appears to be absent as an adaptive feature of frogs from drier habitats.[13]

Early work by Overton (1904), Rey (1937), and Jorgensen (1950c) showed that there is rapid water evaporation from the skin surface of frogs, at a rate comparable with the evaporation from a free water surface. This high rate of evaporation is now thought to be due to the effects of mucus discharge onto the surface of the skin (Lillywhite 1971). Decreased rates of cutaneous water loss were observed where mucous gland discharge was prevented by sympathetic nervous blockade. Decreased rates of evaporative water loss observed in some frogs and toads could therefore be due to low or negligible secretion by the mucous glands, or in Thorson's case (see Footnote) to depletion of the mucus reserves. Thorson (1955) has drawn a striking comparison in rates of water loss between an amphibian, Rana pipiens, and the garter snake Thammophis sirtalis of comparable weight. Under the same conditions, the frog lost weight at a rate more than 40 times that of the snake.

Warburg's (1964) work with anurans and that of Littleford et al. (1947) with salamanders has shown that the vapour pressure deficit is not the only factor involved in evaporative water loss and that other factors, such as

[13] Thorson's (1956) findings may however indicate the existence of a rudimentary water conservation mechanism. Survivors of severe desiccation, when subjected to a second dehydration procedure within a short interval of the first, showed a slight, but consistent decrease in water loss.

high temperature, are important.

More recent work, particularly by ecologists, (Thorson 1955, Claussen, 1969), (Shoemaker 1972) has tended to establish a lack of correlation between terrestrialism and rate of water loss, any apparent differences being due to differences in the size of specimens and to the lack of appreciation of the associated differences in surface area to body weight ratios. There are however, two known exceptions. Loveridge (1970) observed that the Rhodesian frog Chiromantis xerampelina (family Rhacophoridae) lost water very slowly through its dorsal surface which was very impermeable to water. Shoemaker et al. (1972) reported that a South American Anuran Phyllomedusa sauvagii (family Hylidae) had very low rates of evaporative water loss. The rates of water loss were considerably lower than for other anurans tested and were in fact comparable with lizards of similar size.

Two main methods of dehydrating frogs have been used. The apparatus of Thorson and Svihla (1943), Thorson (1956), Schmidt (1965), and Claussen (1969) involved a stream of air passing over a drying material (commonly sulphuric acid or calcium chloride) and then through a chamber of some sort containing the experimental animal. Several of the designs have been critized in that the chamber consisted of a closed jar with a lid permitting the entry and exit of air. Considerable turbulence would have been created and the possibility that the frog was exposed a second time to air that had already passed over it, and picked up moisture, could not be ruled out. Adolph (1932) has shown that frogs lose water at a rate that varies inversely with the relative humidity. Therefore, if the relative humidity in the chamber varies or is elevated, the rate of desiccation will vary accordingly.

Packer (1963), Jameson (1965), and Warburg (1964, 1967), among others, have criticized this approach as bearing little relevance to the general biology of amphibian species. They have followed dehydration in still air,

allowing slower rates of water loss, similar to conditions in the microhabitat where ventilation is generally low.

The latter approach has been used in the present study, with the frogs either in fairly still air and at relatively high humidity (in the room where the frogs were housed) but with no control over temperature, or in climate laboratories (Plant Physiology Division, D.S.I.R.). These climate rooms had plant experiments in progress which dictated conditions. Although temperatures were satisfactory, humidities were too low (15°C, 41% RH, 10°C, 18% R.H.) and so desiccators containing water (95-100% R.H.) or potassium hydroxide solutions adjusted to give a relative humidity of 70-75% (Solomon 1951) were used. However, what gains were made in stable temperature, were more than sacrificed in loss of humidity control, levels in the desiccators changing rapidly when frogs were removed for weighing. Also, the high noise levels and bright lighting, together with the fact that the temporary nature of the perforated aluminium flooring precluded the use of a balance weighing to four decimal places, posed many problems.

Results of trials in the frog housing room only are presented.

ii Materials and Methods

Twenty individuals of each species of similar size and weight (refer to Appendix I) were used in these studies. Groups of frogs, usually eight (four of each species) and of mixed sex [14] were chosen at random and

[14] Sexual differences in general water metabolism are not evident in amphibians although the water content of females during the breeding season is slightly higher than in males because of the large mass of eggs present. Differences in vital limits of water loss between the sexes are also negligible (Thorson 1955). Accordingly, though sex was noted (particularly in Li. ewingi) it has not been taken into consideration.

fasted for several days in containers on wet sphagnum moss and in water to avoid sudden weight losses due to defaecation during a run.

Just prior to an experiment, each frog was removed from its container, rinsed to remove any adhering particles, and the bladder contents expelled by a combination of rhythmic pressure in the posterior abdominal region, and cloacal stimulation. This avoided sudden weight loss due to urination during an experimental run, and gave a more standard comparison by removing an unknown variable (differences in bladder size and contained urine volume in different species) from consideration. The weight of a hydrated frog with an empty bladder is defined as the "standard weight" (Warburg 1964, Claussen 1969).

The animals were then mopped dry with absorbent paper, and were placed in preweighed plastic mesh cages, small enough to limit excessive movement by the frogs, and with perforated polystyrene "doors". The frog and cage weighed to 0.1 mg on a Mettler Balance. The cages were then placed randomly on a wire grid to prevent movement of the cages if the frogs struggled, but which allowed free circulation of air, under and around the cages.

As these experiments were carried out in the room where the frogs were housed, no temperature acclimation was needed. The experiments were carried out in a shaded room with an absence of strong air currents, though the wall grille would have allowed air circulation.[15]

The frog cages were weighed hourly or occasionally at two hourly intervals. The cages were inspected at the time of weighing for traces of urine and on those rare

[15] This was regarded as a compromise situation between experiments carried out at unnaturally high wind velocities and in closed desiccators with unstirred layers of air above the frogs. Conditions were those that both species of frogs may experience in their respective habitats.

occasions where these were seen, test runs for these individuals were terminated and the results discarded. Great care was taken, particularly with the native species, to prevent overdrying of the frogs, after Stephenson's (1961) statement that these frogs are very susceptible to drying and must be protected from this danger if being handled. They were generally dehydrated so as to lose a maximum of 7-8% of their body water. Ambient temperature and humidity were constantly monitored with a recorder calibrated against a centigrade thermometer, and a wet-and-dry bulb thermometer.

Preliminary experiments had shown that the mesh cages could increase in weight in the experimental room if brought in from another room. They were therefore placed in the experimental area an hour before a run and tests showed that they achieved a stable weight within this hour and retained it for the duration of an experiment.

No attempt was made to measure the respiratory water loss from the lungs and this was assumed to be negligible[16] (Thorson 1955) over the time period of an experimental run. The rate of water loss in all cases was based on the actual weight loss by the animals, the validity of this being checked by Schmid (1965) using three different techniques.

Frogs handled at the end of an experimental run rarely urinated, consistent with the findings of Adolph (1927) who reported that amphibians, when removed from water, cease producing urine at once. In most cases therefore, bladder water seems to have been adequately removed.

[16] The relatively low metabolic and respiratory rates in frogs would presumably minimize the amount of water amphibians lost in breathing. Mellanby (1941) could measure no change in the rate of water loss after breathing stopped in the common frog Rana temporaria.

iii Results

Rates of water loss were not constant in the frogs used. Water loss was initially high during the first 1 - 2 hours (refer to figure 4) before settling down to a reasonably constant rate. This was probably due to the fact that, as found by other workers (Warburg 1964, Jameson 1965) not all skin moisture can be removed by the mopping technique. A plot of the rate of water loss (mg/g standard body wt/hr) gave lines of uniform slope after an initial period; the lines were not necessarily in the expected order (on size grounds) above the horizontal axis. This may have been due to the varying amounts of water not removed by mopping, initially setting the relative position of each line. For similar reasons, comparison of percentage body weight lost per unit time is not meaningful. Therefore, the average weight loss (mg/g/body wt/hr) was computed for a 4 - 5 hour period, the results for the first 1 - 2 hours being discarded and the two species were compared on this basis.

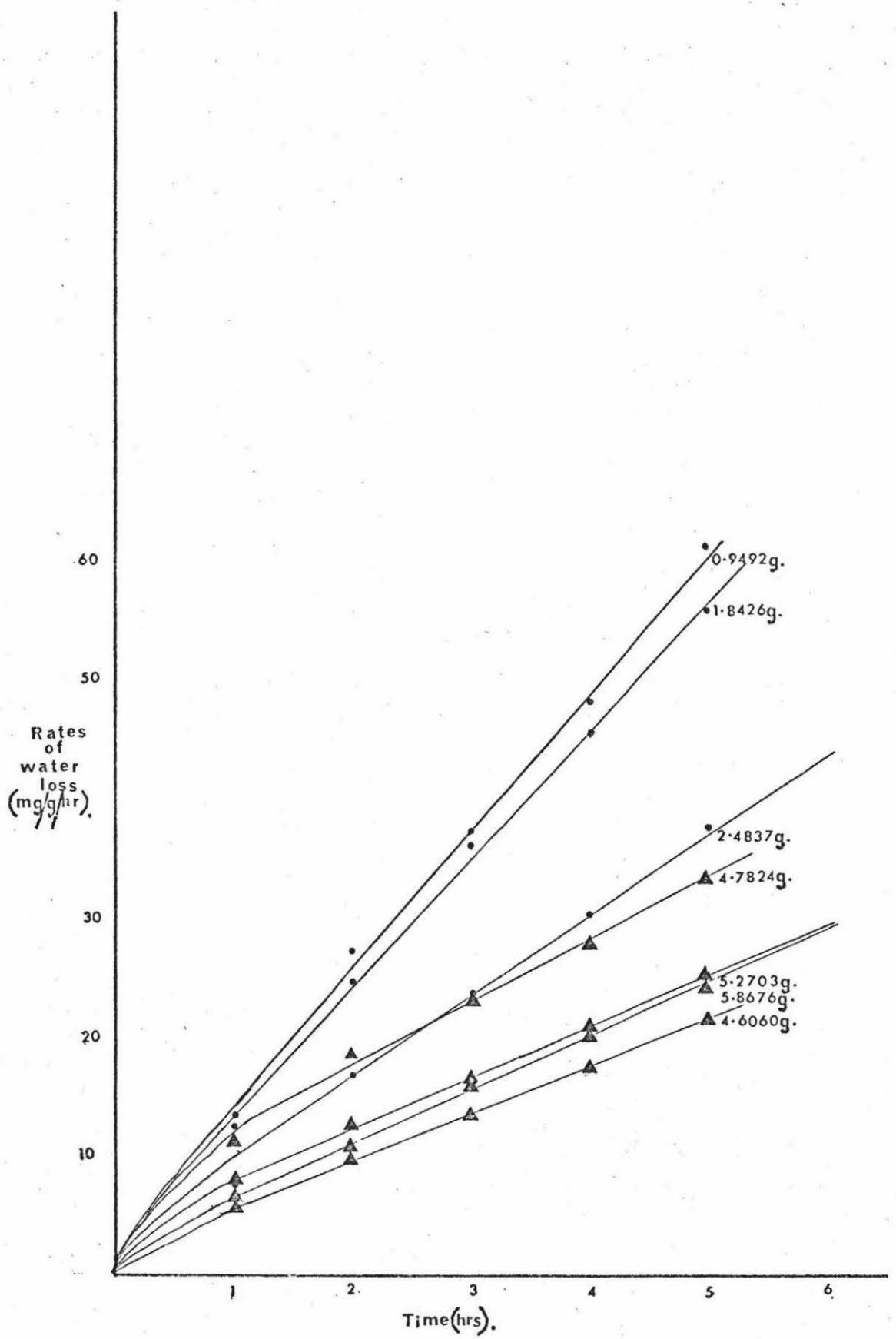
Rates of water loss ranged from 6.9 mg/g/hr (four Li. ewingi, avg wt 3.9 g) and 5.5 mg/g/hr (four Le. hochstetteri avg wt 5.0 g) at 8-5-10°C and 85-87 % RH, to 8.3 mg/g/hr (four Li. ewingi ave wt 3.7 g) and 11.9 mg/g/hr (three Le. hochstetteri avg wt 1.7 g) at 11.5-13°C and 83-85 % RH. (refer to Appendix 2)

The upper histograms in figure 5 (after page 22) are mean rates of water loss for the number of individuals per species per run (usually four). Matching on a weight basis has not been done. The fact that smaller frogs lose disproportionately greater amounts of water than larger ones[17] becomes an important point.

[17] Though smaller frogs lose water at faster rates than larger ones, the converse is also true; they can rehydrate at faster rates than large frogs. They can also tolerate greater losses of body water (Thorson 1955).

FIGURE 4

Typical dehydration results for 2 frog species.
Le. hochstetteri •
Li. ewingi ▲



The lower histograms in figure 5 (after page 22) show more closely matched groups of frogs of both species and no apparent differences are evident. Note however, that comparisons cannot be made between runs from different days due to uncontrolled fluctuations in temperature and humidity.

iv Discussion.

The results generally support the conclusions reached by Thorson (1955), Claussen (1969) that the development of resistance to desiccation has not been an important factor in the appearance of terrestrialism among amphibians.

Figures given by Warburg (1964) for water loss in Li. ewingi (Avg weight 3.4 g - no range given) at 25°C (lowest temperature used) were, under dry conditions (0-5% R.H.) 2.88 mg/g/hr and under humid conditions (95-100% RH) 0.2mg/ghr. My nearest comparable figures for Li. ewingi of similar weight (Avg 3.0 g) would be 6.9 mg/g/hr at 8.5 - 10°C and 85-87% RH in the room.

In fact, my lowest recorded H_2O loss, in a desiccator over water (95-100% RH - checked with cobalt thiocyanate paper and a Lovibond Comparator) is 0.7 mg/g/hr for a single Li. ewingi individual (weight 5.3 g).

However, it is not felt that these differences are important, and merely a reflection of the experimental conditions used. In fact, it appears that Warburg (1964), in using closed desiccators, with little or no air movement, has overlooked the problem of lack of air circulation. Claussen (1969) has also criticized Warburg's results as being very low on the grounds of possible oxygen depletion, CO_2 building up and non constancy of saturation deficit, stating that rates of water loss obtained by Warburg are much lower than obtained in his (Claussen's) study in still air at the same temperature and comparable humidity. No conclusion could be drawn from a comparison of Warburg's data with his own.

FIGURE 5

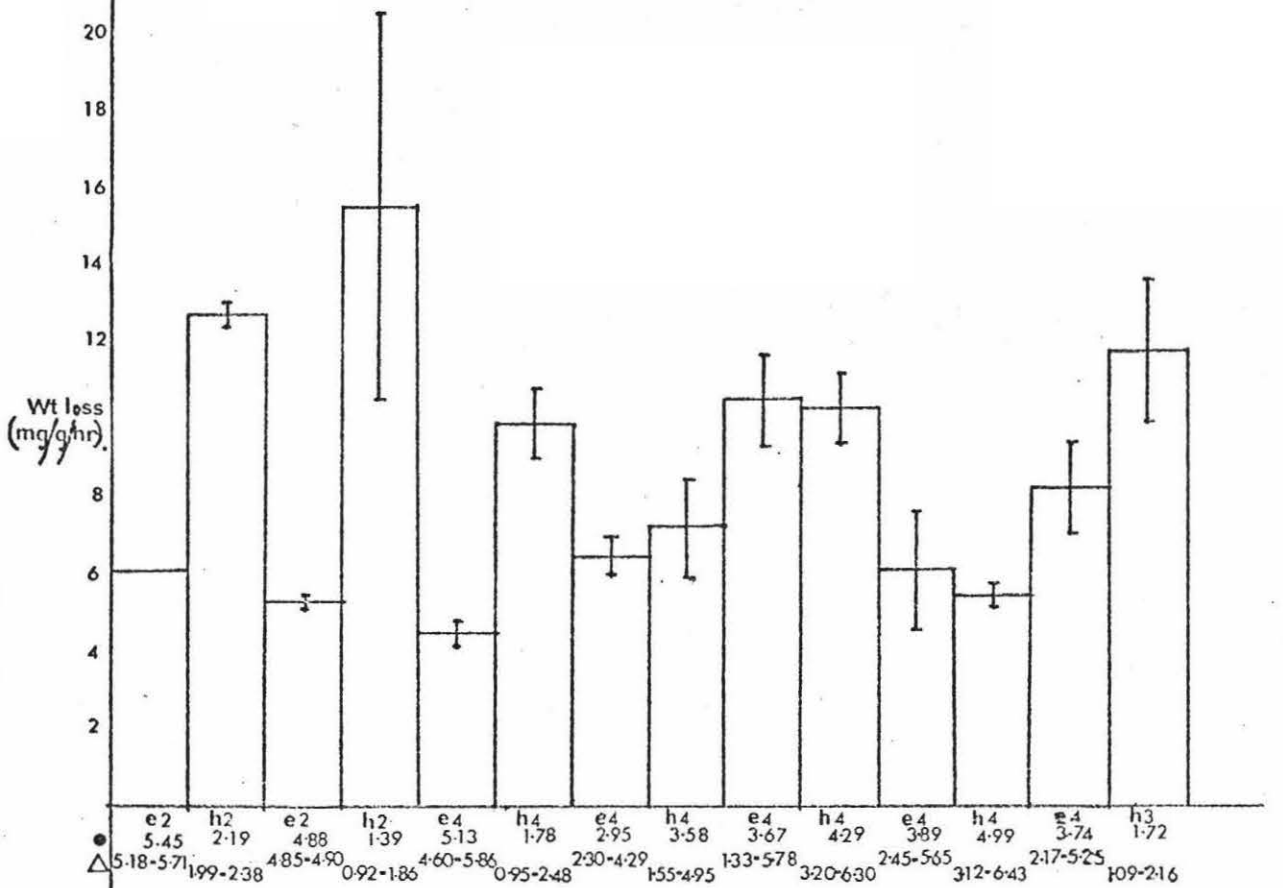
Histograms of average weight loss (mg/g/hr) for indicated number of frogs per species. (±S.E.).

● Mean weight of indicated number of frogs. (g).

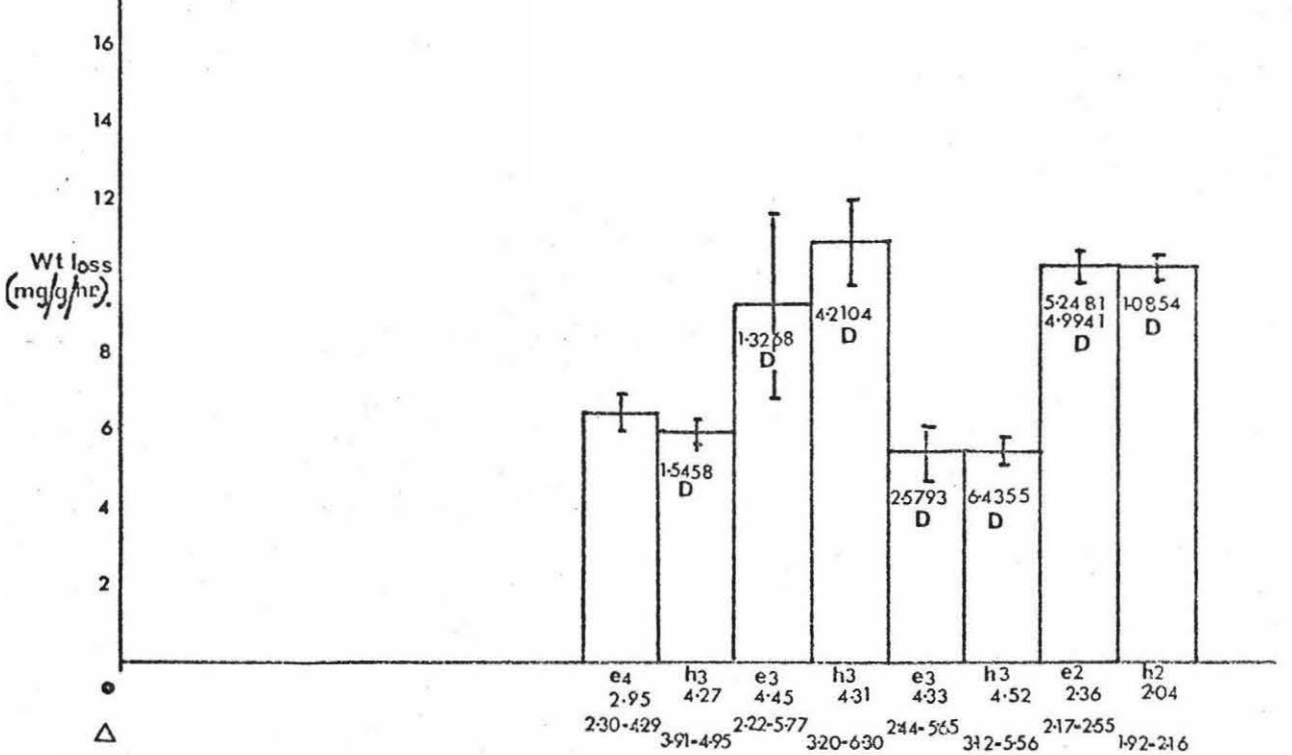
△ Weight range. (g).

e = Li. ewingi

h = Le. hochstetteri



Amended versions of some of the above histograms. Results for selected frogs from different groups deleted to give closer match. (Φ).



Warburg weighed his frogs at two hourly intervals or, at some of the lower temperatures (25°C was the lowest) after 24 hours to six days. This probably accounts for the differences in results that were obtained using desiccators, frogs being removed from their desiccators and weighed at hourly intervals in the present study. This weighing was done in relative humid air. The frogs, in their cages, were returned to their desiccators within 15 seconds, and the relative humidity rapidly attained its former value. No doubt they would have lost considerably less water if weighed after 24 hours.

However, one point remains clear; that there is no detectable difference in the rates of water loss in the two species of frog under the experimental conditions used.

It may be thought that Le. hochstetteri, often surrounded by air of very high humidity, could take up water from this air. Warburg's (1964) results show that frogs in a saturated atmosphere (95-100% RH) continued to lose water, and Adolph (1932) has stated that there can be no uptake of moisture through the skin even in saturated air, the reason being that, when no evaporation occurs, the body temperature is slightly higher than the ambient temperature, thus bringing about a slight loss through evaporation rather than an uptake. This slight loss of body water under humid conditions does not, of course, preclude uptake from rocks and vegetation once water has condensed there. The skin appears to act therefore, like the wick of a wet bulb thermometer at lower humidities, depressing the body temperature of most amphibia below that of their environment (Noble 1954).

This may perhaps explain the finding of Li. ewingi individuals exposed to breezes on a very hot day. In these instances, the temperature of the frogs was probably considerably below that of the surrounding atmosphere. The observations of Harper (in Marriner, 1907) that in Westland, he had noticed the whistling frogs climbing to a height of 7 - 8 feet is interesting. He thought that the frogs only climbed when the surrounding water pools were dry. A

partial explanation at least, may be in terms of temperature control. The development of a dry skin in the early reptiles was an important step in the direction of homoiothermism which the Amphibia failed to follow (Noble, 1954).

It now seems that terrestrial amphibians, rather than being able to prevent excessive water loss are able to tolerate a greater loss of body water than their more aquatic counterparts (Thorson and Svihla, 1943, Thorson, 1955, Schmid, 1965, Gordon, 1965). Body water in amphibians is partitioned into extra cellular (plasma and interstitial fluid) and intracellular components, and it appears that the ability to tolerate desiccation lies in the ability to maintain relatively stable intracellular fluid levels. Smith and Jackson (1931) showed that the tissues of the frog Rana pipiens may lose water differentially. A frog which had lost 53% of its body water, lost only 19% of the water associated with the liver, 37% in muscle, 37% in gut, and 43% in skin.

Essentially similar results have been obtained in toad, while in salamanders, the response is only seen in some tissues, mainly the liver and gut. This retention of water by the tissues is associated with a net accumulation of Na^+ and K^+ ions, presumably followed by the osmotic movement of water (Shoemaker, 1964, Alvarado, 1972).

Lacking an effective skin barrier to water loss, and losing water rapidly in unsaturated air, amphibians would die very quickly if unable to tolerate considerable water loss.

Thorson and Svihla (1943) showed that terrestrial species of anurans tolerated dehydration better than did species from aquatic habitats. The terrestrial toad Scaphiopus holbrookii and the aquatic frog Rana grylio died at losses of about 60% and 38% of their body water, respectively. Littleford et al. (1947) found that for the semi-aquatic salamanders Eurycea bilineata and Desmognathus fuscus, the vital limits of desiccation were

about 15% loss of body weight, while the terrestrial salamander Plethodon cinereus tolerated a loss of about 26%. Vital limits of water loss have not been measured in the frogs used in the present study however, due to limited numbers and unwillingness to subject them to so unnatural and rigorous conditions. They will however, tolerate body weight losses of at least 10% without apparent ill effects.

A good correlation between the degree of adaptation to life on land and survival at high temperatures has also been reported by some workers.

v Behavioural Observations.

Many previous investigators have failed to observe the actions of amphibians undergoing dehydration; these may be important (Bentley et al. 1958, Packer 1963, Deyrup 1964), as is perhaps best reflected by the statement of Bentley (1966) that amphibians have "used their heads" to find favourable micro environments for survival and reproduction. Some workers have also explained anomalous water exchange data of at least one frog species (Heleioporus) in terms of burrowing behaviour.

Frogs of both species made initial escape attempts when placed in their cages for dehydration and these movements may have contributed to the initially high rates of water loss. These attempts became generally less frequent after the first 1-2 hours. Thereafter, the whistling frogs would hunch up in a crouched position in their cages with arms and legs pressed close to, and tucked under, the body. They appeared to be trying to protect the exposed ventral surface. The native frogs though, would merely sit or crouch in their cages, arms and legs spread apart, with seemingly little effort to protect the ventral surface. These postures are remarkably similar to those described by Barwick (1961).

Sporadic escape attempts did persist but were

only successful twice. In both cases, native frogs were involved (recaptured) and had managed to push partially or completely through unnoticed small gaps in the plastic mesh.

Whereas the whistling frogs would stand up and place fingers and toes in the plastic mesh, or struggle quietly in the cages, the native frogs would close their eyes and try to force their heads through any opening during these escape attempts. Considerable force was often used, the neck and body bending under the pressure, with no apparent ill effects.

Generally, the native frogs appeared more robust than the specimens of Li. ewingi of similar size and had generally more muscular thighs and flattened bodies. This may be correlated with the habits of the native frogs - burrowing into crevices among rocks and squeezing through narrow gaps in rock and foliage. This is well illustrated by the disappearance of one of the smaller specimens from its aquarium; despite intensive searches in the rocks and debris of the aquarium, it could not be found. In desperation, a small rotting tree branch lying in the cage was closely examined. In one end a small hole leading into the branch was almost dismissed as being too small to accommodate a frog. However, something shiny could be seen and, careful prodding and poking coaxed out a lively little frog. It appeared head first and must have therefore originally backed a considerable distance down this hole which was barely big enough for the frog, and certainly provided no room for turning.

Li. ewingi species merely crouched aerially in their aquaria, in the shelter of flax leaves, or in little depressions in damp sphagnum moss or earth.

(b) Water Uptake Studies.

i Introduction.

Several workers have found that the rate of water passage through the skin, both in normally hydrated and in dehydrated amphibians is related to the habitat.

Terrestrial amphibians have the highest rates of uptake while aquatic species have the lowest rates. (Jorgensen, 1950c - cited by Deyrup, 1964; Main and Bentley, 1964; Warburg, 1964; Schmid, 1965; Bentley, 1966). Uptake of water by frogs after a period of dehydration has also been studied by Thorson (1955), who could not relate the rate of water uptake after prolonged periods of dehydration to the terrestrial nature of some species. He noted in his paper though, that Overton (1904) reported that a six gram tree frog Hyla arborea, after a loss of 1/3 of its body weight through desiccation, regained its water at a rate 25 times that of Triton cristatus of the same size, or a toad Bombinator igneus of similar size under the same conditions. Overton ascribed the tree frogs ability to remain out of water, regaining its evaporated water from dew on leaves, to the great absorbing power of its skin. Bentley et al. (1958) have found a higher rate of water uptake after dehydration in several species of Neobatrachus that are well adapted to life in semi-arid habitats and these findings are confirmed by Warburg (1964).

Rey (1937) compared the rates of uptake in hydrated animals of five species and found that the terrestrial species absorbed water more rapidly than aquatic types. Bufo vulgaris and Salamandra maculosa, terrestrial toads and salamanders respectively, absorbed water at rates four to five times those of the more aquatic Triton marmoratus and Rana esculenta. Jorgensen (1950) obtained essentially the same results using Bufo bufo and Rana temporaria, although dissimilar experimental conditions resulted in somewhat different absolute values.

It is assumed that weight increases are solely due to flow of water through the skin at a rate dependent on the osmotic gradient between the two sides of the skin (Sawyer, 1951) and to a minor extent on equivalent amounts of water following active uptake of Na^+ ions through the

skin. There is general agreement that dehydrated amphibians do not drink when given access to water (Adolph, 1927a, Thorson, 1955, Gordon *et al.* 1961),[18] and in fact they do not need to due to the relative ease with which water passes into these animals through the skin. As the animals were fasted during these experiments, water ingested with food can also be ignored.

It would be of considerable survival value if those frogs living in drier habitats, some distance from ponds and free surface water, could take up moisture in the form of rain and dew rapidly, when it appeared. This high rate of natural water uptake would confer a certain independence from water for considerable periods of time, but would, on the other hand preclude a predominantly aquatic existence for these frogs due to the continual "swamping" of the body with water and overloading the kidneys trying to "bail out" this excess water.

Xenopus laevis an aquatic toad with some primitive anatomical features (Noble, 1954) has been shown to have little or no increase in rates of water uptake after dehydration, whereas in *Bufo regularis* the rate of water uptake increased by three to seven times that of control levels (Ewer, 1952b).

[18] However, the rather obscure paper by Schlumberger and Burk (1953) apparently overlooked by most investigators is rather disturbing in this regard. These workers report that contrary to statements in the literature, frogs do swallow water. After a frog was kept for 24 hours in 0.1% colloidal thorium dioxide in distilled water, roentgenograms showed the colloid in the gastrointestinal tract. The authors further state that because the frog's skin is impermeable to colloids the conclusion appears justified that the thorium dioxide was swallowed. The fact that frogs can be induced to drink, particularly when placed in salt solution hypertonic with respect to the body fluids may not therefore apply here and the problem of whether or not frogs do drink under normal conditions may require re-examination. Schlumberger and Burk also found that calcium (in water) is absorbed through the intestinal mucosa rather than through the skin.

Parallel with these observations, it was found that when neurohypophyseal peptides were injected, Bufo retained large amounts of water, while Xenopus completely failed to respond (Bentley, 1971). It is also relevant that elimination of the source of octapeptides by destruction of the hypothalamic preoptic nuclei, increases urine volume and reduces cutaneous water absorption in dehydrated toads (Shoemaker and Waring 1968).

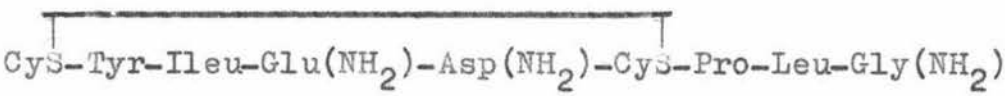
Few urodeles have been examined for their ability to rehydrate following dehydration but Spight (1967b) has measured this in four species of salamanders and found only relatively small increases in rates of water absorption. Urodeles also only accumulate water slowly after being injected with neurohypophyseal peptides (Bentley, 1971).

It would seem therefore, that the amphibians, like other tetrapods are aided in their osmoregulation by the action of some hormones, and the most important of these are secreted by the neurohypophysis, and the adrenocortical tissues.

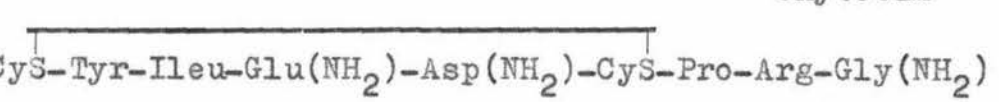
In the amphibia, for the first time in the vertebrate series, there is found a separate circulatory supply to a specialized part of the neurohypophysis, the pars nervosa; the Amphibia therefore have the first anatomical basis for direct release of hypothalamus-derived materials into the systemic blood (Gorbman, 1964). It is also pointed out by Gorbman that the neurohypophysial substances so released are important in physiological water conservation, and the Amphibia, being the first terrestrial vertebrates, are the first animals to encounter the need for water conservation. An interesting point is that in the dipmoan fish (lung fish) which face this need, there is a partial anatomical organization of this kind (Wingstrand, K.G., 1959). In addition, the aquatic salamanders with a lesser or no need for water conservation, have a less well developed hypophysial portal system than do the anurans.

Early work by Brunn, (1921), (reported by Bentley, 1971), showed that when extracts from the neurohypophysis of mammals, which have an anti-diuretic action in this group, are injected into frogs kept in water, there is an increase in weight due to an accumulation of water. This action has been called the Brunn effect or "water balance effect". Heller, (1941) found that the pituitary of Rana temporaria contains a substance causing anti-diuresis when injected into rats though this was not identical to the activity present in the mammalian neural lobe. The amphibian extract was far more potent in its ability to produce a "water balance effect" when injected into frogs than were similar mammalian extracts. This amphibian extract was found to be arginine vasotocin also present in birds, reptiles and most fishes, but absent from mammals. A second substance with oxytocic properties (8 - isoleucine oxytocin or mesotocin) was also found to be present in amphibian neurohypophysial extracts. Oxytocin has been identified in the neurohypophysis of a single amphibian Rana pipiens, but mesotocin is also present in this frog (Bentley, 1971).

Vasotocin, mesotocin and oxytocin can all promote water retention in various amphibians but vasotocin is easily the most active of these peptides (Bentley, 1971). There appears to be no vasopressin, at least in the species tested (Gorbman, 1964).



Oxytocin



Arginine Vasotocin

Molecular structures of Oxytocin and Arginine vasotocin.

The only physiological action which has been clearly identified with the neurohypophysial hormones in the

Amphibia is related to the movement of water and salts through certain membranes (Jorgensen, 1950; Ewer, 1952; Gorbman, 1964), and the effect on water metabolism is produced by responses at three known sites:- the mesonephros, the skin, and the urinary bladder. All of these three responses are directed towards the counteracting of dehydration. It is interesting that fish have these same hormones in their neurohypophyses, but they do not respond to them in any of the three modes in Amphibia i.e. reduction of urine volume, increased skin permeability and net reabsorption of fluid through the bladder wall. The water conserving action of the hormones is reinforced by the fact that aquatic amphibians are less responsive to neurohypophysial hormones than terrestrial amphibians; aquatic tadpoles, although their pituitaries contain anti-diuretic hormone, are relatively unresponsive to it until they approach metamorphosis (Gorbman, 1964).

Injection of neurohypophysial peptides can reduce the urine volume in a large number of anurans and urodeles, though this response may be poor or undetectable in some amphibians, especially aquatic forms like Xenopus laevis and Necturus maculosus.

The action of vasotocin in reducing urine volume is a dual one; it depresses the glomerular filtration rate, and increases the renal tubular reabsorption of water, though the tubular response appears to predominate (Sawyer, 1957a). The locus of the hormonal action is the distal part of the nephron and only "osmotically free" water is involved (water which would move in response to osmotic differences between the blood and urine if the intervening membrane permitted).

It has been proposed that an increase in pore size of the distal tubular cell membranes is responsible.

Vasotocin and the related peptides from the neurohypophysis increase the permeability of the skin of anurans to water, the only known exceptions being Xenopus

laevis and the crab eating frog Rana cancrivora (Bentley, 1971). The skins of a variety of urodeles, have been examined for this response but none of them have been found to react in this way to such peptides. These findings agree with those for Xenopus which, along with the urodeles, rehydrates more slowly than most anurans, presumably because there is only a renal effect.

It appears that in the skin too, only the movement of osmotically free water can be accelerated by the hormones as, if the external medium is isosmotic or hyperomotic with respect to the inside medium, the hormone does not stimulate water movement. There is good evidence therefore, that the neurohypophysial hormones increase the relative permeability of the skin to water and the work of Sawyer (1960b) indicates that reabsorption of water from the urinary bladder of frogs follows the same principles. It appears however, that under minimal levels of circulating hormones, the kidney is far more responsive than the skin. Gorbman reports the work of Buchborn (1956) who found that the first renal response was obtained at a dose level 1/75 to 1/100 of that required for the skin response. This suggested to Gorbman that the hormonally stimulated skin transfer of water occurs only under extreme circumstances.

Since Gorbman (1964) voiced the opinion that the in vitro skin permeability response to water after application of neurohypophysial hormones was very minor rate to kidney response and of doubtful significance in the intact animal, more recent in vivo work (McLanahan 1969) Christensen (1974) has shown that ventral skin permeability does increase markedly during dehydration and similar findings have been assumed to be due to hormonal mediation by many previous workers. In fact, Bentley (1971) makes the important point that vasotocin has been found to increase the permeability of the skin in Rana esculenta even when present at the concentration of only

10^{-10}M and that vasotocin is released into the blood of dehydrated frogs and toads in which it is present at a concentration of 10^{-9} to 10^{-10}M. This is thought to be adequate to produce skin permeability increase in dehydrated anurans. However, Barker - Jorgensen and Rosenkilde claimed in a discussion on a paper presented by Sawyer (1954), that denervation of the neurohypophysis leading to complete inactivation, gave no difference in water uptake in response to dehydration in Bufo bufo when compared with control animals. In a later paper Christensen (1974) refers to two previous investigations where this work is reported more fully (Jorgensen, et al. 1969, Christensen and Jorgensen 1972). Jorgensen et al. investigated the role of the preoptic nucleus and its axons following transection of the neurohypophysis, on the assumption that it may have taken over the function of the treated tissue. The results did not however, indicate the functioning of the preoptic nucleus. Extirpation of the neurohypophysis seemed to prevent the anti-diuresis and increase in permeability to water of the urinary bladder which normally follows osmotic loading or strongly reduced blood volume. Conversely, cutaneous and renal responses to dehydration were found to be about equally pronounced in toads with extirpated neurohypophyses as in toads with completely eliminated preoptic-neurohypophyseal systems. Christensen and Jorgensen (1972), continuing this work found that total hypophysectomy or extirpation of the pars distalis significantly reduced the increase in cutaneous water permeability in the response to dehydration, salt loading and vasotocin. In contrast to the cutaneous response, the anti-diuretic response to salt loading showed greater dependency upon the presence of a functioning pars nervosa than upon the pars distalis. They concluded that the increase in cutaneous water permeability in response to increased osmotic pressure of the blood is not dependent upon a functioning pars nervosa, but upon the pars distalis, and that the pars distalis

does not act by directly increasing the water permeability of the skin but by permitting the action of the permeability increasing factors including vasotocin. If the pars distalis has only a permissive role though it is rather puzzling that the cutaneous response in dehydrated animals is still seen after extirpation of the neurohypophysis unless some functional tissue remained or the neurosecretion material which accumulates proximal to the cut could still be released. The mediation by other hormones must obviously be considered.

The experimental observations of Ewer (1952a) Sawyer and Schisgall (1956), and Shoemaker (1964) have shown that frogs and toads absorb water from their urinary bladders when they are dehydrated. It has also been shown to occur in at least one urodele (Ambystoma tigrinum). However, this would only happen to an appreciable degree in Ambystoma during dehydration when the blood would become increasingly hypertonic with respect to the urine and water would be drawn osmotically across the bladder wall and back into the general circulation, maintaining a relatively constant internal mileu. The relatively large urinary bladder in amphibians, a structure which has no homologue in their phyletic forebears the fishes, is able to store relatively large amounts of water (though this varies between species). In the absence of stored urine, amphibians in air have no means of altering the increased concentrations of body fluids resulting from evaporative water loss. The effects of evaporation tend to be minimized though because of the high water content and low concentrations of body fluids typical of hydrated individuals. Dilute urine stored in the bladder can be utilized to maintain normal levels of plasma Na^+ until water losses equivalent to the volume of urine initially present in the bladder have occurred (Shoemaker, 1964).

Though not measured, it appeared that Li. ewingi specimens did void considerably more urine (e.g. before

dehydration runs) than did Le.hochstetteri individuals.

Neurohypophysial peptides increase the osmotic permeability of the bladder of a variety of anurans including members of the families Ranidae, Bufonidae, Hylidae and Leptodactylidae but not of the Pipidae (Xenopus) (Bentley, 1971) and vasotocin has been shown to be particularly effective (Sawyer, 1960). However, only one species of urodele is known to respond (Salamandra maculosa) while the urinary bladder of Ambystoma, Necturus, Triturus or Siren and Amphiuma shows no increase in osmotic permeability (Bentley, 1971).

Two factors are operating here then. In fully hydrated frogs water uptake may be greater in those species from more terrestrial habitats, and, as a further adaptive feature, this rate of uptake and rate of water reabsorption from kidney and bladder may be considerably augmented in dehydrated animals by specific pituitary hormones. The neurohypophysial content of substances implicated in water and electrolyte regulation is reducing during dehydration in frogs and toads (Levinsky and Sawyer, 1953; Jorgensen et al. 1956). Though some kind of blood volume receptor may be involved, an increase in the solute concentration of body fluids appears the most likely stimulus for release of the hormone. Injections of hypertonic salt solutions (which would increase body fluid volume) are the most effective stimulants for neurohypophysial hormone release (Gorbman, 1964).

Many workers, when studying rates of water uptake in amphibians, have ligated the cloacas after removing the bladder water. The frogs have then been immersed in water for varying periods and reweighed, the ligature preventing any weight loss through urination.

Attempts to ligate the cloacas of some of the whistling frogs proved unsuccessful. This technique was not tried on the native frogs due to the limited numbers and the fear of damaging the cloacal skin and causing

death. The frogs were therefore handled carefully, particularly after periods in the water and when mopping (to remove surface water), before weighing.

ii Materials and Methods

(a) Water uptake in hydrated animals.

Four frogs of each species (Li. ewingi and Le. hochstetteri) were fasted for several days in containers of wet sphagnum moss, and were weighed after removal of bladder water. They were then placed in individual plastic dishes containing water to such a level that only the head of each frog was above the water. Petri dishes at an angle in these containers ensured that the frogs remained in the water, but were still left with an air pocket.

The frogs were carefully removed from their containers after 1 hour, mopped dry with absorbent tissue, and reweighed. Bladder water was then expelled and the frogs were replaced in the water for another hour. The average weight increase per hour was thus obtained. These determinations were done on the eight frogs in the same day, at 10-12°C.

(b) Water uptake after dehydration

The same two groups of four frogs were dehydrated for a certain period calculated so that they lost 10% of their body weight. Rates of water loss to 10% cannot be compared in the two groups as the temperature and humidity varied over the two days in which these experiments took place (refer to table 1).

The frogs were weighed hourly and observed closely during the 6-8 hours that it took to lose the required weight. They were then placed in water under the same conditions as in (a) and weighed, after mopping, at hourly intervals in their cages which were weighed in

parts (a) and (b) just prior to each frog weighing, to allow for any water in the cages. The cages remained at a constant weight however.

No attempt was made to remove the bladder water after each weighing here, as the passage of water into the animals was of interest, whether this was passing into the tissues or into the bladder. The results obtained are artificial of course in that, under dehydrating conditions "in the field" the frogs would have variable amounts of water stored in their bladders which could be withdrawn through the bladder wall into the general body circulation as dehydration proceeded. They probably would not lose this amount of water normally either, but would react behaviourally in a potentially desiccating environment by leaping to a more suitable place.

iii Results (Refer to table 1, figures 6 and 7.)

Rates of rehydration in hydrated frogs ranged from 80 mg/hr for four Li. ewingi of mean weight 3.64 g (28.9 mg/g/hr or 2.2% of body weight per hour) though rates as high as 5% per hour were recorded for an individual of body weight 2.33 g, to 29 mg/hr for four Le. hochstetteri of mean weight 5.07 g (6.05 mg/g/hr or 0.57% of body weight per hour) and as high as 0.8% per hour for an individual of body weight 3.15 g. Rates of water uptake (mg/g/hr) were generally higher in smaller frogs of both species.

These results indicate a rate of rehydration under conditions when the animals are fully hydrated that is different for each species; water uptake is approximately 2.8 times as rapid in Li. ewingi as it is in Le. hochstetteri on a mg/hr basis.

Rates of water uptake after dehydration show a dramatic increase over the hydrated values (particularly in Li. ewingi) although these are not directly comparable

TABLE 1

Rates of water uptake in two species of frogs.

(a) Hydrated uptake.

(b) Uptake after dehydration. (10% body weight loss)

	Hydrated		After Dehydration	
	Body wt (S.W.) gm.	Mean uptake (mg/hr)	Mean uptake (mg/gBW/hr)	Mean uptake (mg/hr)	Mean uptake (mg/gBW/hr)+
Li. ew.	2.3323	121.9	52.27	315.9	140.14
	1.8968	57.55	30.34	389.3	207.10
	4.8844	65.25	13.36	761.0	154.30
	5.4282	76.10	19.99	589.7	107.10
Mean	3.6355	80.20	28.99	513.98	152.16
S.E.	± 0.89	± 14.4	± 8.5	± 100.6	± 20.8
Le. hochs.	6.3296	39.0	6.16	82.15	13.59
	3.1579	26.55	8.41	99.40	33.28
	5.5563	26.3	4.73	79.6	15.06
	5.2484	25.6	4.88	77.35	15.28
Mean	5.0731	29.36	6.05	84.63	19.30
S.E.	± 0.68	± 3.2	± 0.85	± 5.0	± 4.6

+ g.B.W. is the standard body weight before dehydration.

Note. Test weighings determined Li. ewingi weighings accurate to ± 3mg and Le. hochstetteri weighings accurate to ± 5mg.

FIGURE 6

Rates of water uptake for 2 frog species. (Mean ± S.E.)
Li. ewingi (Avg wt 3·63g.).
Le. hochstetteri (Avg wt 5·07g).

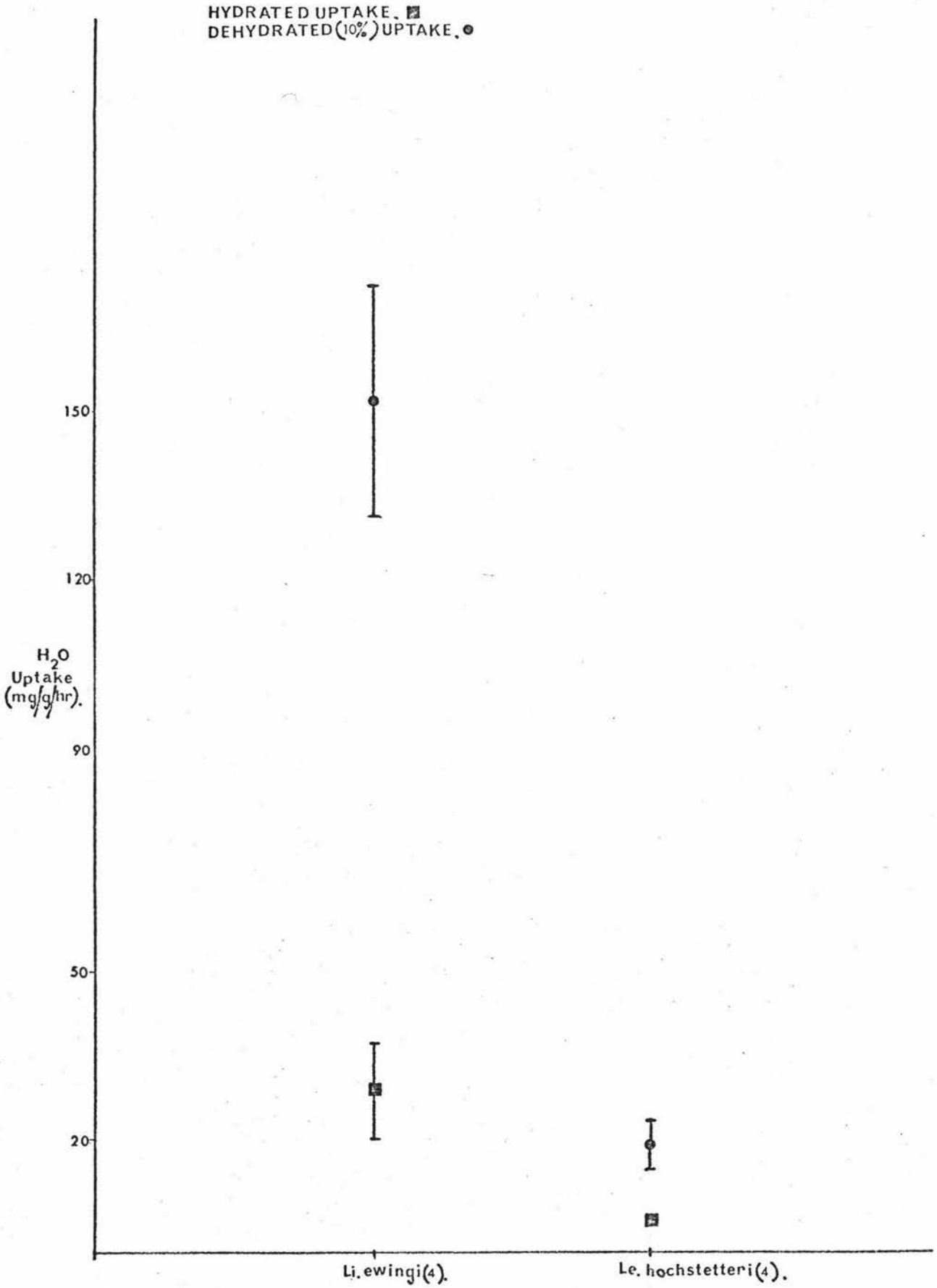
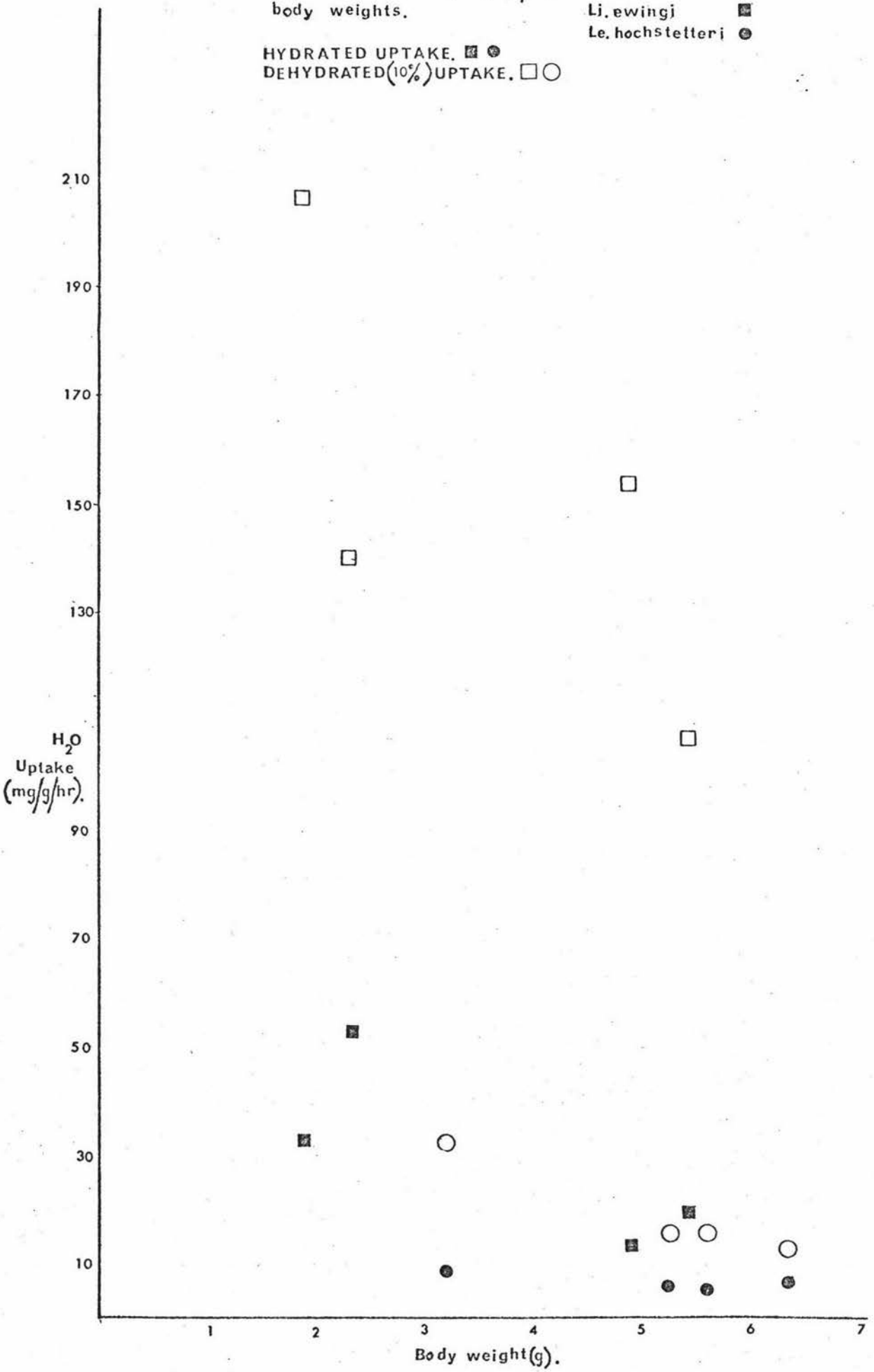


FIGURE 7

Rates of water uptake (mg/g/hr) for frogs of different body weights.

Li. ewingi ■
Le. hochstetteri ●

HYDRATED UPTAKE. ■ ●
DEHYDRATED (10%) UPTAKE. □ ○



due to temperature differences. (refer to figure 6)

Rates of water uptake ranged from 513.9 mg/hr (mean rate for four Li. ewingi) or 152 mg/g/hr (14% of body weight per hour) and as high as 20% per hour for an individual of body weight 1.89 g, to a rate of 84.6 mg/hr (mean rate for four Le. hochstetteri) or 19.3 mg/g/hr (1.66% of body weight per hour) and as high as 3% in an individual of body weight 3.16 g. Rates of rehydration in dehydrated Le. hochstetteri do not even approach rates of water uptake in hydrated Li. ewingi. The greater variability shown by Li. ewingi was probably due to their rapid rehydration and the loss of some of this water by urination.

At the end of the first hour in water (to rehydrate) after dehydration, all Li. ewingi individuals were observed to have quite large amounts of water in their bladders. As they had more than attained their original (hydrated) standard weights at this stage, the rehydration runs were terminated, and when they were replaced in their holding containers, they invariably climbed up and crouched on the walls. The impression gained from this was that, having replenished their body water at a high rate, they were not immediately able to "switch off" the mechanism that had caused it. They therefore stopped the high rate of water uptake by actively getting out of this medium.

Sawyer (1954) states that the effects of injected neurohypophyseal hormones last for several hours, gradually wearing off after this time. Endogenous hormones might be expected to act similiarly.

Rates of water uptake were much slower in dehydrated Le. hochstetteri, taking several hours to attain the hydrated standard weight, and these frogs remained in water in their holding containers at the end of this time.

iv Discussion

Warburg (1964) has studied rates of rehydration in dehydrated frogs in Australia. The frogs in his study were dehydrated by an amount equivalent to 15-20% of their body weight for periods ranging from 24 hours to 6 days, and were then placed in water at 20°C. Rates of water uptake in the species he studied ranged from 120 mg/g/hr for species of Neobatrachus centralis (average weight 11.7) a burrowing frog from arid habitats, to 105 mg/g/hr for species of Crinia signifera (average weight 1.2g), a species remaining close to water throughout their life, and 42 mg/g/hr for species of Li. ewingi (average weight 3.4 g), also reported by Warburg as remaining close to water throughout their lives. His methods are not clearly described, however, and he may have removed bladder water between weighings. Main and Bentley (1964) also did this. However, as rates of water uptake through the skin will primarily determine the rate of incorporation into the body fluids and tissues, (whether directly or from the bladder) bladder water was not removed in the frogs used in the present experiment.

Warburg's values for Li. ewingi are much lower than obtained here, so supporting the above suspicions. Assuming that he did not remove bladder water between weighings, possible reasons for the difference were looked for. These could include temperature, seasonal differences in the levels of neurohypophysial hormones, the effect of stress, differences in geographic range and in the rates of dehydration.

In Rana pipiens, an increase in temperature of 10°C increases the rate of water passage through the skin 2.3 times (Noble, 1954). Bentley (1971) has also found that water transfer across the skin is influenced by temperature. The Malayan toad Bufo melanosticus, when kept in water at 12°C, gains water through the skin at a rate of 6.4/ul/cm^2/hr and at 29°C this is more than doubled (17/ul/cm^2/hr).

Considering that in the present study control values were obtained at 10-12°C (Similar to the temperature in the room where the frogs were housed) while rehydration data were obtained at approximately 16°C for Li. ewingi and at approximately 17.5-19°C (on the following day) for Le. hochstetteri, the effect of temperature is bound to be significant. Values at these higher temperatures would be considerably reduced at 10-12°C. Mean water uptake in dehydrated Le. hochstetteri at 17.5-19°C (in mg/hr) is 2.9 times that at 10-12°C for hydrated frogs. (refer to table 1) If, therefore, this rate has been increased up to 2.3 times by temperature, rates of rehydration do not become much higher than the control values. However, rate of water uptake in Li. ewingi at approximately 16°C is 6.4 times that at 10-12°C. Even allowing for temperature effects, rates of rehydration would still be considerably higher (3-5 times) than control values.

Possible differences in the seasonal levels of circulating pituitary hormones affecting water balance (Gorbman, 1964) could also be important, particularly in the specimens of Li. ewingi. Deyrup (1964) reports the work of Uranga (1957) who found that anti-diuretic activity was much higher in summer than in winter in the parts of the brain of Bufo arenarum which gave this reaction. The present data were obtained during late spring and early summer, but Warburg (1964) does not supply this relevant information for his frogs.

The possibility that the mild rates of dehydration (though higher than in Warburg's studies), and the relatively high temperature could have "stressed" the animals must also be considered. Adrenalin has been shown to increase in vivo the osmotic permeability of the skin of toads Bufo melanosticus (Elliot, 1968) and frogs in vitro (Jard et al. 1968) (reported by Bentley, 1971). If this hormone was released into the circulation during dehydration (presumably a stressful experience) it could add to

the action of the neurohypophysial peptides or act alone. It is now known that cAMP mediates at least some effects of the adrenal hormones and the hormones of the neurohypophysis (Bentley, 1971). The rates of dehydration may also have caused greater stimulation of the neurohypophysis through a rapid rise in blood osmolality in Li. ewingi with a correspondingly greater increase in hormone release. The rise in blood solute concentration could account for at least part of the increase in water uptake in both frog species though it is probably masked at least in Li. ewingi by the hormonal response. Also, the reported effects of the hormones on the kidney and bladder would result in the lower likelihood of urination during rehydration as more of the water taken up through the skin would be retained in the body to make up the deficit.

It may also be that the particular population or populations of frogs studied by Warburg (1964) do not show the whole range of adaptations to life in moist to drier conditions, and his statement that Li. ewingi remain close to water throughout their life tells little of the specific conditions under which they were caught. In fact Warburg, using a similar argument to explain some of Thorson's (1955) results, states that it is already well known, particularly with regard to frogs, that populations from different localities may be adapted to temperature to varying extents and also possibly to humidity.

Such differences have been found between populations of Hyla rubella from north-western Australia and from eastern New South Wales, as well as in Limnodynastes tasmaniensis from a mesic habitat in the Mt Lofty Ranges in South Australia, and from a xeric habitat in the semi-arid upper Eyre Peninsula in South Australia.

Marriners (1907) comments are also interesting. He states that Li. ewingi is a true tree climbing frog but according to a Mr J.J. Fletcher (who had done much work on Australian frogs at that time) it had, at least

in Australia, altogether or nearly lost the arboreal habits of a tree frog. He further stated that in Westland, it still seemed to do a fair amount of climbing to a height of 6-8 feet. Marriner could not decide whether the abundance of bush in Westland had stimulated the frogs to make use of a power it had almost lost in Australia but thought that environment in Westland was certainly conducive to this activity. This suggests that the frogs may also have developed another power they had almost lost in Australia - that of rapid water uptake; this would be of considerable value to tree frogs but not necessarily to ground living and water loving frogs.

The fact that the initial attempt to introduce Li. ewingi into the North Island from Westland was not successful suggests that perhaps conditions were close to one extreme of the range of these frogs with regard to some undefined factors.

Thorson (1955) has shown that Rana pipiens (a semi-aquatic species) of average weight 20 grams, when desiccated to a loss of 28% of their body weight, regained their original weight in 2.8 hours at 22°C. Claussen (1969) has compared rates of water uptake in six species of anurans representing four genera (Bufo, Scaphiopus, Hyla and Rana). They ranged in weight from 6-20 g (Bufo punctatus) 7-16 g for B. debilis, 8-25 g for B. boreas, 15-25 g for Scaphiopus couchi, 13-15 g for Hyla septentrionalis and 40 g for Rana Catesbeiana. Temperature also fluctuated in these experiments to some degree. The animals were desiccated at 25°C to 80-85% of their standard weight after removal of bladder water. Bladder water was not removed between weighings during rehydration.

The calculated time for rehydration from 90 to 100% standard weight was, in increasing order, 0.39 hours for H. septentrionalis, 0.59 hours in R. catesbeiana, 0.64 hours for B. boreas, 1.22 hours in B. punctatus, 1.12 hours in S. couchi and 1.49 hours in B. debilis.

Rates were considerably higher initially in rehydrating from 80-85% of their standard weight at least

in B. boreas and H. septentrionalis with values falling from 40-12% standard weight gained/hour (in the first hour) in B. boreas and 37-10% standard weight gained/hour (in the first hour) in H. septentrionalis. In agreement with Thorson (1955) however, Claussen could find no obvious correlation between habitat selection and rate of rehydration. Though the various anuran species he tested showed significant differences in rates of rehydration, he concluded that these differences do not necessarily represent adaptations to terrestrial conditions.

However the present results may reflect a true adaptation, in that Le. hochstetteri may be limited to wet areas because of its primitiveness whereas Li. ewingi may possess modifications enabling a more terrestrial life.

Consideration of the smaller size of the experimental frogs used in this study makes it likely that the rates of water uptake measured could be expected, at least in Li. ewingi. Rates of uptake were measured over the first hour in Li. ewingi and over the first two hours in Le. hochstetteri, while Claussen weighed his animals at 15 minute intervals.

The higher rates of water uptake through the skin in Li. ewingi compared with Le. hochstetteri, even in fully hydrated animals, may, in part at least, explain why some deaths were experienced when Li. ewingi were kept under wet conditions. Schmidt (1965) found that although amphibians from terrestrial habitats tolerated a significantly greater loss of body water during desiccation, they did not survive the prolonged stress of hydration as long as did aquatic species, and he has suggested that those species which exhibit low tolerance to hydration are not able to winter in water.

Frogs specialized for a particular habitat may therefore be limited to it (and precluded from existence in others) through physiological specialization.

5. ISOLATED SYSTEMS

(a) Introduction.

Because rates of water gain in Li. ewingi are considerably higher than in Le. hochstetteri when both frogs are in a fully hydrated state, and this difference is magnified on dehydration, it was decided to investigate those factors contributing to these differences.

Schmidt (1965) studied the water economies of nine species of amphibians. From Fick's Law of Diffusion, he deduced that the influx of water into a species is a function of the permeability of the skin of that species to water, and the internal concentration of dissolved particles, assuming that the environment is relatively constant in its concentration of dissolved materials.

Accordingly, measurements were made of the blood plasma osmolality and the skin permeability in the two frog species under study, and in a third (L. aurea) known to be able to exist in water for long periods of time (refer to details of habitat, and housing).

Also, it was decided to test the effects of two neurohypophysial hormones on the isolated skin to see whether the response was correlated with increased rates of rehydration after desiccation (particularly in Li. ewingi) and whether temperature effects could explain most of the increase in Le. hochstetteri. (Assuming there would also be some renal response). Because of the limited number of frogs available, and the realization that such small frogs would have to be sacrificed for blood sampling, techniques were used that enabled the measurement of several parameters. Where skin potentials and short circuit currents could have been measured using two perspex half-cells, small glass tubes with flared ends were used instead so that permeability measurements could be made on the same skin section.

(b) Osmolality of the Blood Plasma.

i Introduction.

The concentration of materials in body fluids of frogs with variously permeable skins will influence the rate of passive water uptake through this skin because of the osmotic gradient it imposes.

Amphibia in water have the problem of water inflow, as their plasma osmolality is above that of the surrounding medium (water) and their skin is comparatively permeable. It would be an advantage to them to lower their plasma solute concentration, thus reducing the osmotic inflow of water tending to "swamp" them and which they must "bail out". Alternatively terrestrial frogs might be expected to have a higher plasma osmolality as this would aid the inflow of water when it became available. It would also enable a urine of higher concentration to be produced, (there is no evidence that this exceeds the blood concentration in amphibians though) (Alvarado, 1972) and would allow some water conservation, urine of a higher concentration requiring or containing less water than more dilute urine.

Water makes up approximately 80% of the total body weight of most amphibians, though this may vary between species (77.4% in Pseudacris nigrita, 79.9% in Hyla crucifer, 80.3% in Hyla versicolor, 83.1% in Rana clamitans and 83.5% in Rana pipiens), (Schmidt, 1965). This is a higher proportion than the 70% seen in most other tetrapods and the osmotic concentration of the body fluids is usually less (200-250 milli Osmoles) than that of other tetrapods (300 m.Osm) (Bentley 1966). There is less sodium present in amphibian plasma (about 115 milli moles/litre) than in other terrestrial vertebrates (about 150 m.moles/l) (Bentley, 1961), which accounts for most of the difference. However, levels of plasma sodium may vary considerably, depending on species, habitat, osmotic condition, and food intake.

Amphibians, even more than reptiles though, have a considerable ability to tolerate different solute levels in their body fluids (Bentley, 1971). Whereas man has difficulty surviving a concentration of sodium in the plasma of 170 m. moles/l (330 milli osmoles) frogs and toads can recover from body fluid concentrations double that which is considered normal (Bentley, 1961). The amounts of water loss or metabolic solute accumulation consistent with life are great, compared with those for other terrestrial tetrapods. Also as mentioned previously, water is reabsorbed from the bladder so that plasma solute changes little during dehydration until the reserve is exhausted.

ii Materials and Methods.

The frogs were kept in conditions designed to duplicate as closely as possible their natural habitat. (refer to section on housing). They were caught, handled as little as possible, and transferred quickly to the laboratory. Each animal was then stunned, double-pithed and laid out on Ringer moistened paper tissue. The abdominal area was irrigated at intervals with aerated frog Ringer.

Because of the small number of suitably sized Le. hochstetteri available and the overriding concern previously expressed, a mid-ventral incision through the skin, underlying muscle and pectoral girdle, to expose the heart was not attempted. Rather, a cut was made as far anterior as practicable (usually along the base of the buccal cavity) the scissors were then turned posteriorally on both sides and the skin was cut along the sides of the body. The skin was then carefully reflected to half way down the trunk and irrigated with frog Ringer at frequent intervals. The mid ventral incision was then made, exposing the heart. The heart was freed of its pericardium, removing this source of resistance to the passage of the needle, and

allowing greater manipulation of the heart.

A straight pair of forceps was initially used to grasp the apex of the ventricle but this often led to tearing of the heart muscle when the needle was being positioned. A pair of curved forceps was therefore slid round behind the base of the heart and the needle pushed into the ventricle against this support. A number 27 needle on a disposable 1 ml plastic syringe (non heparinized) was used to practise blood sampling. This proved no problem with L. aurea specimens, where their relatively large size allowed ease of entry into the ventricle and rapid withdrawal of blood into the syringe.

However, in the other two species, much smaller in size and with correspondingly smaller hearts and reduced blood volume, some problems were encountered in obtaining the necessary volume of plasma (0.05 ml) required for the measurement of blood osmolality. Therefore, rather than trying to transfer the sampled blood from syringe to heparinized (Lithium heparinate) micro-haematocrit tubes, this step was omitted and ventricular pumping used to fill the tubes with blood. The heparinized tubes were drawn to a fine point at one end over a spirit burner. This area was then bent gently causing a sharp jagged break in the thinner section. This then served as a hypodermic needle and enabled exact location of the tip within the ventricle, as the rate of entry of blood into the tube could be readily observed. Two full tubes (or 3-4 partially filled ones) were obtained in this way. The other end of each tube was plugged with plasticene, and samples were centrifuged for 3 minutes on an International Micro capillary centrifuge model MB.

Care was taken to handle the tubes only about the middle region to prevent the possible contamination of the whole blood or plasma with Na^+-rich human sweat. They were then filed and snapped at the junction of the plasma and blood cells. The sharp end was also usually removed.

The plasma was then forced into a 0.05 ml measuring vessel by blowing the contents out of the tube with a modified syringe. Osmolality (mOsm/kg) was then measured on a Knauer Electronic Semi-micro osmometer. Two measurements were usually made on each sample.

Samples were then diluted for ion determinations. Undiluted or 100 times diluted plasma was stored in sealed vials for up to one week in a refrigerator before ion analysis was done on a series of samples.

Na^+ and K^+ determinations were carried out on an E.E.L. flame photometer and Cl^- determination was done on a Buchler-Cotlove Chloridometer. Duplicates were prepared in all cases.

All glassware was rinsed repeatedly with tap water (6 times), glass distilled water (6 times) and oven dried. The standards (500 ppm Na and 1000 ppm K) were stores in polythene bottles and diluted as needed with glass distilled water. The cloride standard for titration at low rates (0.5 m Molar NaCl) was stored in a soda glass bottle and used as required.

iii Results (refer to table 2 and figure 8)

The mean value for plasma osmolality is higher in Li. swingi (234 m osmoles/Kg) than in L. aurea (222 m osmoles/Kg) though the mean plasma Na^+ value is somewhat higher in L. aurea and plasma K^+ and Cl^- values (m M/l) are similar.

However, the lower mean Na^+ value in the plasma of Le. hochstetteri is reflected in the markedly lower osmolality (183 m Osm/Kg). Plasma K^+ and Cl^- values are also lower than in the other two species (refer to table 2).

iv Discussion

Plasma ion values and osomolalities are in reasonable agreement with other figures given for frogs (Deyrup, 1964; Bentley, 1971). Plasma Na^+ and Cl^- values are quite

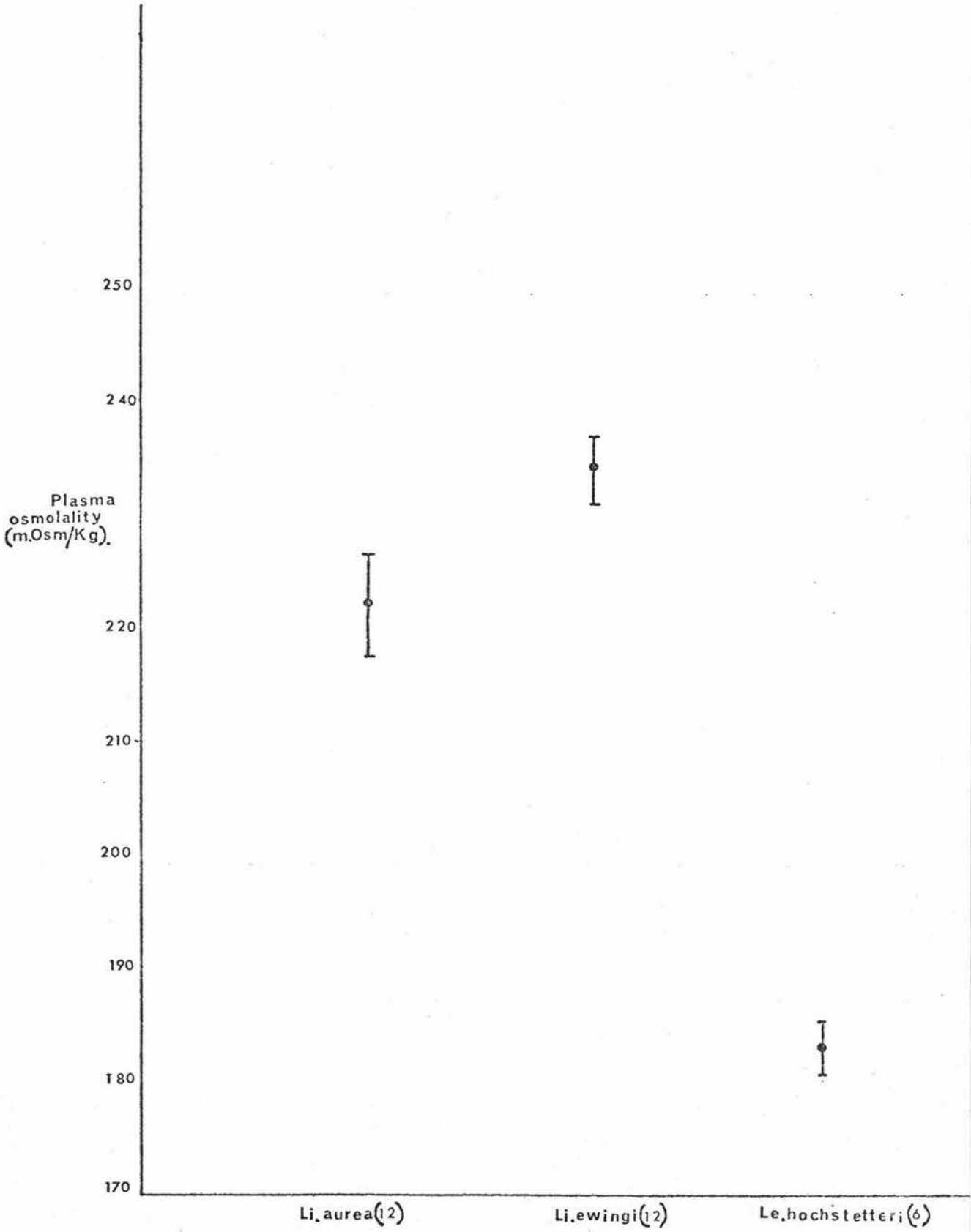
TABLE 2

Blood Ion Composition (m.M/l) and Plasma osmolality (m.Osm/l) of Three Frog Species

Plasma Na^+	Plasma K^+	Plasma Cl^-	Plasma Osmolality
Litoria aurea (12)			
108.3	2.9	73.4	233
113.0	4.0	52.9	204
117.4	4.3	70.8	236
98.7	4.5	73.3	205
115.2	4.2	65.5	215
126.1	4.8	75.1	242
123.9	5.5	89.8	236
115.2	3.2	78.3	220
96.9	4.1	84.1	234
110.0	4.5	63.7	198
93.0	3.9	86.5	232
104.3	4.2	73.7	212
Mean			
110.2	4.2	73.9	222
S.E.			
±3.0	± 0.2	± 3.0	± 4.4
Litoria ewingi (12)			
93.5	4.7	75.6	224
98.3	4.1	81.5	212
99.1	3.3	70.6	228
100.0	3.3	70.6	240
96.9	4.1	69.9	246
104.3	3.5	76.0	239
93.5	4.3	77.4	225
101.3	4.4	72.7	229
			237
			244
			245
129.13	5.19	92.7	241
Mean			
101.8	4.1	76.3	234
S.E.			
±3.8	± 0.23	± 2.6	± 3.0
Leiopelma hochstetteri (6)			
98.3	3.2	54.2	176
96.5	4.7	51.9	182
94.3	3.7	38.3	192
83.5	3.4	52.9	183
			178
90.8	3.4	50.1	186
Mean			
92.7	3.7	49.5	183
S.E.			
±2.6	± 0.25	± 2.9	± 2.4

FIGURE 8

Blood plasma osmolality (m.Osm/Kg.)* of 3 frog species.



* Mean ± S.E. of indicated number of frogs.

variable for reasons previously indicated and the effects of handling could also be quite important.[19]

The values for Le.hochstetteri are considerably lower than in the other two frogs, particularly the plasma Cl^- values and the osmolality. Alvarado (1972) has analysed the body fluids of the urodele Ambystoma tigrinum (tiger salamander) which spends most of its adult life living in burrows under logs and in other moist places and his values are not very different from those in the two Australian frogs used here (Na^+ 112 m moles/l, Cl^- 84 m moles/l, total solute concentration 231 m moles/l). The data for urodeles are generally scanty but the values given for Necturus maculosus are in better agreement with present results for Le. hochstetteri (Na^+ 84.8 - 97.47 m M/l, K^+ 3.2 - 3.6 m M/l, Cl^- 66.6 m M/l).

Comparing Li. ewingi and Le. hochstetteri, there is a considerable difference in plasma osmolality which reflects the differences in water uptake in animals of both species. The contribution of plasma proteins to plasma osmolality should not perhaps be underestimated. Feldhoff (1971) found that the albumina of Rana catesbeiana plasma increased throughout metamorphosis. Total protein increased and the absolute amount of albumin increased more than tenfold. The overall increase in albumin level was explained in terms of the transformation of an aquatic tadpole into a terrestrial frog requiring a more efficient vascular system and a more sophisticated osmoregulation.

Urea accumulation during dehydration has been

19 There may be significant losses of Na and Cl^- ions through the skin of amphibians subjected merely to rinsing or gentle handling (Bentley, 1958a; Deyrup, 1964, or by the "pricking" effect (an increase in chloride loss following any injection) Sawyer, 1954).

shown to occur in the aquatic anuran Xenopus laevis and in the Urodele Ambystoma tigrinum (Delson and Whitford 1973). These workers have suggested that this mechanism was used by early amphibia in resisting water stress, by the storage of nitrogenous wastes as urea and thus reducing urinary water loss. The same mechanism could be operative in Le. hochstetteri and the other two native species. McLanahan (1972) has also pointed out that the same mechanism reduces an unfavourable osmotic gradient between an amphibian and a dehydrating environment.

(c) Skin Permeability

i Introduction

As terrestrial frogs lose water as rapidly as aquatic anurans of comparable size, (Thorson, 1955; Claussen, 1969) it may be thought to be of selective advantage for them to take up water rapidly when this became available as rain or dew.

Schmid (1965), using leg skin bags, found that the skin of aquatic species of anurans was less permeable to water than that of terrestrial species. Spight (1967a) found that the six species of salamanders studied by him could absorb water from a soil sample that contained about 10% water. When the moisture content was lower (3-4%) the salamanders slowly lost water. However, it was apparent that the salamanders would be able to absorb water from the soil in the agricultural areas in which they live.

Dole (1967), investigating the role of substrate moisture and dew in the water economy of leopard frogs, Rana pipiens, found that previous workers studying water balance in anurans had given little attention to determining the mechanism by which these animals obtain water under natural conditions. Apparently assuming that leopard frogs depend on ponds or other bodies of water for their moisture, investigators had used free water to supply these frogs with their needs. Dole, however, noticed during

field work that in summer, adult leopard frogs are nearly completely independent of bodies of water, though usually confined to moist places. He found that they depend on soil moisture as their primary source of water, and absorb it through the skin of the groin. Leopard frogs dehydrated to 65-75% of their hydrated weight could, on the average, completely recover the lost water in 48 hours when on sand with a moisture content of 20%. He further found that dew, absorbed through the groin by sitting on wet vegetation, could be used as a supplementary water source but was apparently not necessary if sufficiently moist soil was available. Dole makes the further point that, even in very arid habitats, dew is often formed during the night and may be a significant source of moisture for anurans inhabiting such areas. Certainly, it is likely that many arboreal species depend upon dew as their main source of water (Dole, 1967).

McLanahan and Baldwin (1969), following Dole's (1967) findings, and observing that Bufo punctatus emerged from rocky desert canyons to rehydrate by pressing against moist rocks, thought that there may be some specialization of the ventral pelvic integument permitting the rapid absorption of water. Consistent with this behaviour, it was found that in dehydrated toads, the ventral pectoral integument took up insignificant quantities of water, but the ventral pelvic integument was found to take up water at an average rate of 423 mg/cm^2/hour. This rate of uptake could account for approximately 70% of the rate observed across the total integument. These findings confirm the earlier results of Hevesey et al. (1935), who found that the skin of the abdomen and thighs in amphibia is more permeable than the webs of the feet.

Extending this work, Bentley and Main (1972) have compared the osmotic permeability of, and active sodium transport by different parts of the skin from five anuran species from contrasting geographic regions, ecological

niches and systematic groups; experiments were conducted both in the presence and absence of vasotocin. Zonal differences in the permeability of these skins were found to exist in some species, which Bentley and Main thought may be of physiological significance. They also report the findings of Loveridge (1970) who observed that the Rhodesian frog Chiromantis xerampelina lost water very slowly through its dorsal surface which was very impermeable to water, but was able to readily absorb water across its protected ventral skin.

Under dehydrating conditions, neurohypophysial peptides (particularly vasotocin in amphibia) are thought to facilitate rapid uptake of water through the skin. Bentley et al. (1958) found that four species of Neobatrachus showed considerable differences in their abilities to absorb water after dehydration; species from drier areas gained water at a faster rate than those from wetter areas. Such differences in ability to rehydrate were paralleled by the rates at which these frogs accumulated water after being injected with a neurohypophysial peptide oxytocin.

Considering the above facts and the water uptake data in hydrated and dehydrated frogs, it was decided to measure dorsal and ventral skin permeability in the absence and presence of oxytocin and vasopressin (ADH).

ii Materials and methods

The method used was essentially that of Bentley and Main (1972). This enabled simultaneous measurement of permeability, electrical potential and short circuit currents of the dorsal and ventral skin. (Electrical data will be considered in a separate section.) As large an area of dorsal and ventral skin as possible was removed from individuals of the three frog species, cutting carefully through the connective tissue delineating the lymph sacs. The skin sections were then placed in a dish of

aerated frog Ringers solution of the following composition: (mM/l) NaCl 111, KCl 1.88, $CaCl_2$ 0.82, NaH_2PO_4 0.0008, $NaHCO_3$ 2.4, distilled water to 1000 ml. Glass tubes were used, flared at one end (internal diameter 0.85 cm, external diameter 1.1 cm) and with glass arms near the other end over which a loop of wire could be secured for weighing purposes. The flaring process had caused irregularities in the ends of the tubes and were ground to give a plane surface.[20]

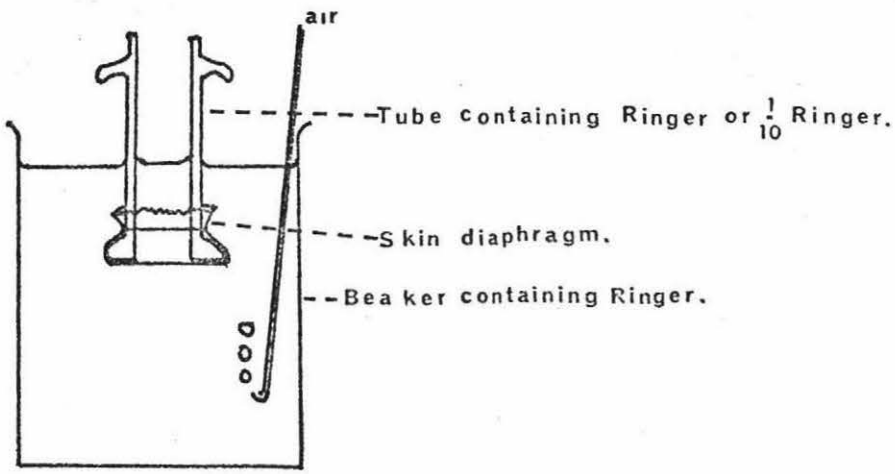
A strip of parafilm was wound round the outside of each tube near the flared base where the skin diaphragm would be tied onto the tube on the assumption that the cotton used in tying would "bite" into the underlying parafilm, providing a good seal. The skin sections (dorsal and ventral) were then tied on to separate tubes with the inner skin surface facing outwards (and therefore bathed by the Ringers solution in the beakers) and the outer surface facing into each tube and the excess skin was cut off, as close to the cotton as feasible. The tubes, complete with skin, were each then placed in 50 mls of aerated frogs' Ringer solution in beakers, and filled to approximately the same level inside with full strength frog Ringer by means of a plastic cannula on a syringe (refer to figure 9).

The preparations were then allowed to settle down for one hour. The inner solution was replaced at intervals during this time. The potential difference (P.D. in millivolts) was taken periodically until a constant value was attained. Short circuit current values (S.C.C. in μ amperes) assumed to reflect active sodium transport were then recorded.

[20] Bentley and Main (1972) used the flared ends of test tubes, but due to problems in cutting this type of glass, test tubes were not used in the present study.

Figure 9

Skin Diaphragm Preparation
(for osmotic and electrical
measurements).


air
Tube containing Ringer or 1/10 Ringer.
Skin diaphragm.
Beaker containing Ringer.

To study the effects of oxytocin and vasopressin on the P.D. and S.C.C. values, the Ringers solution bathing the inner skin surface (the solution in the beaker) was replaced with fresh Ringers containing the appropriate concentration of the hormone (10m U/ml).

Then, in both cases (hormones absent or present) the internal solution in each tube was replaced several times with 1/10 Ringers solution (diluted with distilled water). The tubes (and skin) were removed from their beakers and mopped with absorbent paper tissue to remove excess solution from the tube and the surface of the skin facing outwards. Also, drops of fluid resulting from the plastic cannula in contact with the inner wall of the tubes were removed by mopping with a small cylinder of tissue. The tubes were then weighed to 0.1 mg and replaced in the beakers containing the aerated solutions. They were removed again, usually after one hour, mopped and reweighed to measure weight loss.

The preparation could be tested for leaks and the goodness of seal at the end of an experiment by placing a wet finger over the open end of a tube, and watching the skin bulge out at the other end. Another finger could then be placed on this bulged out section and pressure applied. Invariably, there was no pressure loss.

Water movement through the skin of frogs occurs down a gradient of osmotic concentration. When the solutions on either side of the skin are of equal concentration, little water is transferred. This "non osmotic" or electro osmotic water movement (Kirschner *et al.* 1960) is thought to be associated with active ion uptake (e.g. Na^+) to maintain osmotic equality, and is normally minor (Bentley, 1971). This movement of water would be indirectly increased by neurohypophysial hormones, due to their direct action in stimulating Na^+ uptake. However, the main movement of water across the skin is directly proportional to the osmotic gradient between the two sides of the skin, and is considered to be a passive

process, not requiring metabolic energy (Bentley, 1971). There is evidence (Davson, 1970) that passage of water across such membranes as toad skin or toad bladder under an osmotic gradient is largely a function of flow through pores rather than by simple.diffusion through the membrane.

iii Results

(a) Skin permeability to water under the influence of an osmotic gradient
(refer to table 3 and figure 10)

The dorsal skins of the three species show similar permeabilities, though the mean value for Li. ewingi is higher than for the other two species, perhaps reflecting the thickness of the skin which appeared to be less than in the other two species.

The ventral and dorsal skin of Le. hochstetteri exhibited similar permeabilities but in L. aurea and Li. ewingi the ventral skin permeability was 2.5 times and 4.8 times, respectively that of the dorsal skin in these frogs.

(b) Skin permeability to water under the influence of an osmotic gradient, in the presence of oxytocin or vasopressin
(refer to table 3 and figure 11)

While oxytocin and vasopressin had no appreciable effect on dorsal skin permeability of the three species tested, the ventral permeabilities were affected to differing degrees. No permeability change was observed in the ventral skin of Le. hochstetteri if the result from frog four is discounted (refer to note at bottom of tables 3 and 4). Oxytocin and vasopressin both increased ventral skin permeability in the other two species though oxytocin was more effective than vasopressin in both cases (refer to table 3).

TABLE 3

Skin permeability (mg/cm^2) of dorsal and ventral skin of three frog species to water under the influence of an osmotic gradient. (Full strength Ringer bathing inner skin surface; 1/10 Ringer bathing outer skin surface.)

(a) Unstimulated.
(b) In presence of oxytocin 10 mU/ml. (ox).
(c) In presence of vasopressin 10 mU/ml. (vp).

Litoria aurea

	Permeability Dorsal	+ ox	+ vp	Permeability Ventral	+ ox	+ vp
1.	2.32			7.26		
2.	4.84			6.21		
3.	1.58			7.79		
4.	2.63			5.89		
5.	2.00			5.58		
6.		2.21			44.74	
7.		3.26			54.84	
8.			2.95			42.00
9.			2.74			41.47
Mean	2.67	2.74	2.85	6.55	49.79	41.74
S.E.	± 0.57	± 0.53	± 0.10	± 0.42	± 5.1	± 0.26

Litoria ewingi

1.	4.63			27.26		
2.	2.84			13.94		
3.	7.79			28.42		
4.	3.79			11.16		
5.		3.58			102.0	
6.		4.32			115.61	
7.		3.05			185.79	
8.			2.42			49.53
9.			4.32			46.53
10.	2.84			23.86		
Mean	4.38	3.65	3.37	20.93	134.5	48.0
S.E.	±1.0	±0.37	±0.96	± 4.0	± 26.0	± 1.5

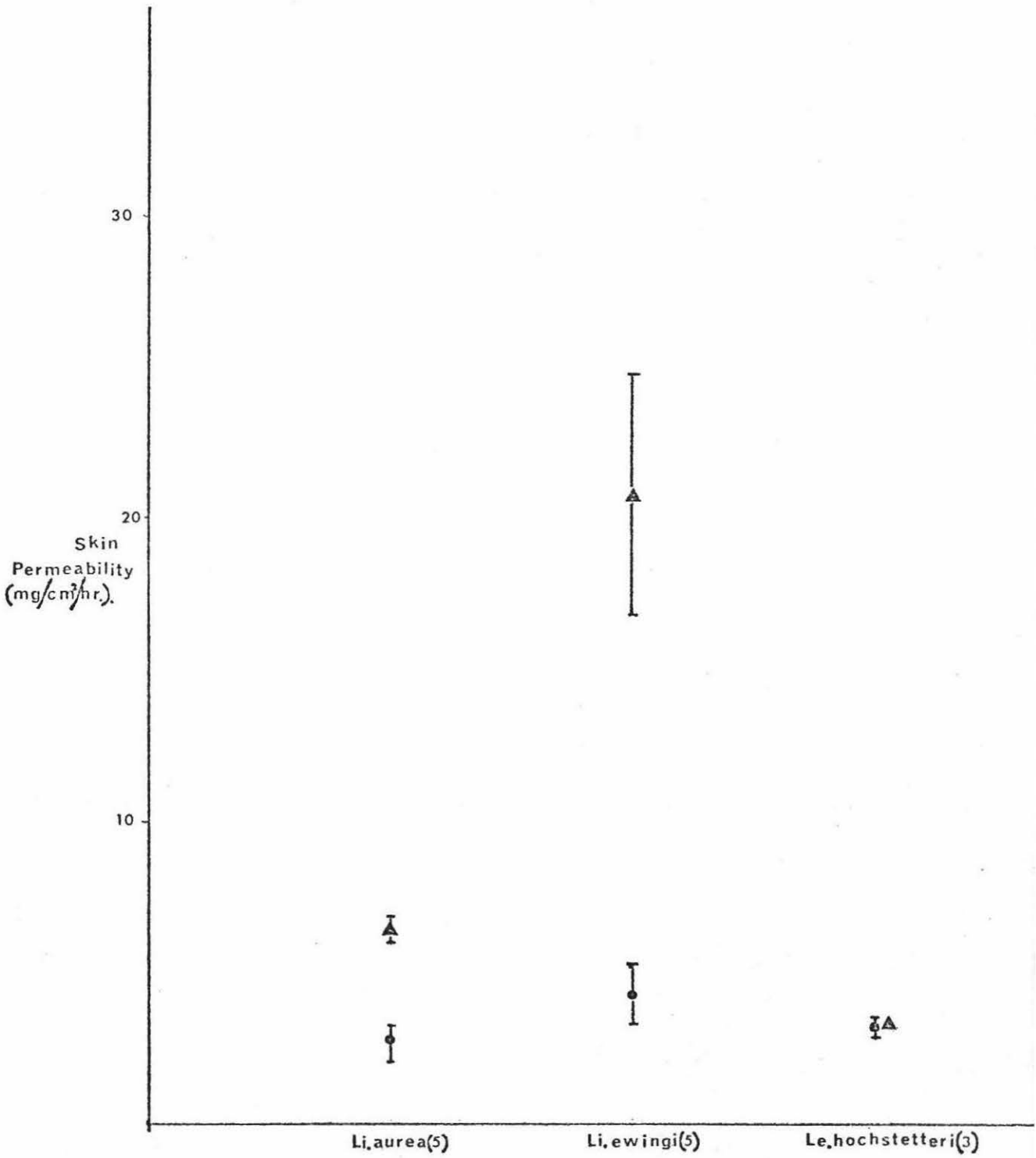
Leiopelma hochstetteri

1.	2.63			3.37		
2.	3.79			3.37		
3.	3.47	3.05		3.47	3.79	
4.		4.53			12.84 *	
5.		3.47			3.89	
6.			3.16			3.89
Mean	3.30	3.68	3.16	3.40	3.84	3.89
S.E.	± 0.35	± 0.44		± 0.1		

* May be a weighing error, or due to a leak.

FIGURE 10

Skin permeability ($mg/cm^2/hr.$)* of dorsal and ventral skin of 3 frog species under the influence of an osmotic gradient.



* Mean ± S.E. of indicated number of frogs.
● Dorsal skin.
▲ Ventral skin.

FIGURE 11

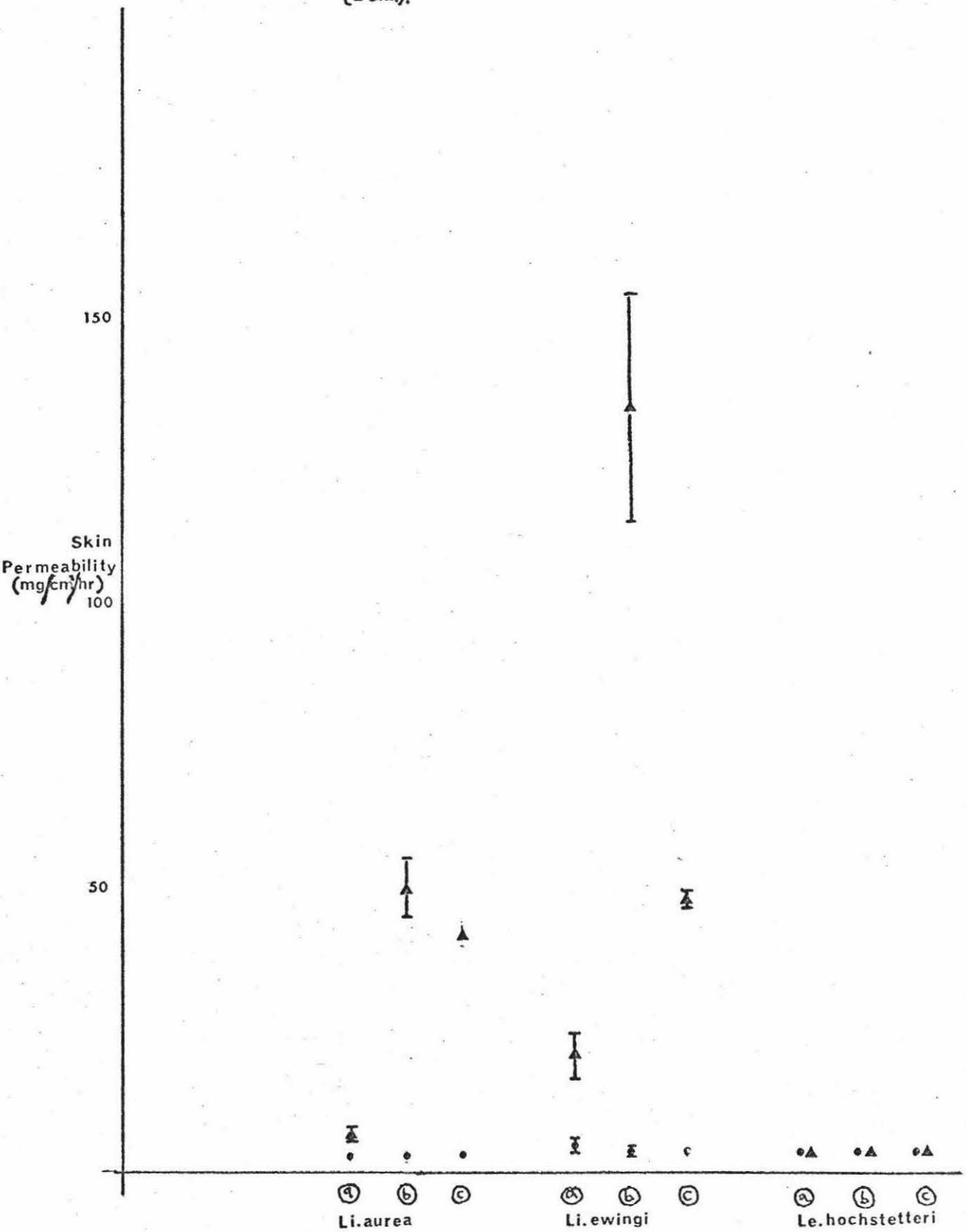
Skin permeability (mg/cm²/hr) of dorsal • and ventral ▲ skin of 3 frog species under the influence of an osmotic gradient.

ⓐ Unstimulated.

ⓑ In the presence of oxytocin (10mU/ml).

ⓒ In the presence of vasopressin (10mU/ml).

(± S.E.).



Oxytocin resulted in a 7.6 times increase in the permeability of the ventral skin in L. aurea and a 6.4 times increase in the permeability of the ventral skin in Li. ewingi (though as high as 8.9 times in one individual) giving average values of water gain of 134.5 mg/cm^2/hr for Li. ewingi, 49.8 mg/cm^2/hr for L. aurea and 3.84 mg/cm^2/hr for Le. hochstetteri.

iv Discussion

Many observations have been made on the differential responses of amphibians to mammalian neurohypophysial hormones. The skin and kidneys of toads are much more sensitive to vasopressin than to oxytocin whereas in frogs the sensitivity is greater to oxytocin (Deyrup, 1964). The results for the permeability of the ventral skin of the three frog species in the absence of hormones generally agrees with the findings of Schmid (1965), in that the skin of the aquatic L. aurea was less permeable to water than that of the more terrestrial Li. ewingi. The predominantly aquatic nature of Le. hochstetteri is also indicated. Schmid (1965) used leg skin bags,[21] and at least in some of his species the full extent of the permeability differences would have been masked because the permeability results would have been the sum of the collective permeabilities of dorsal and ventral leg surfaces. The permeability of the dorsal leg skin would be similar to that of the dorsal body surface and so, greater permeability differences may have resulted, particularly in the Hylids, had ventral skin exclusively been used. The present results may also explain the ability of L. aurea to remain in water for long periods, and the differences in water uptake in the two other species.

[21] His results are considerably above those of Bentley and Main (1972) and cannot be compared.

Although the skin of L. aurea does show some permeability specialization it is much less pronounced than in Li. ewingi, and would present a more effective barrier to water influx. It may be, that on reinhabiting water these frogs have not fully lost their more terrestrial adaptations. The ventral skin of Li. ewingi is considerably more permeable than that of Le. hochstetteri and L. aurea which may at least partially explain its more terrestrial and arboreal existence. The apparent lack of specialization of the ventral skin of Le. hochstetteri probably reflects its aquatic habitat. This wasalso found for Xenopus laevis (Bentley and Main, 1972) and no response has been found in a variety of urodeles that have been tested with these peptides (Bentley, 1971). It has been suggested that the increasing sensitivity of target sites (kidneys, skin) may have played a part in amphibian adaptation to life on land (Ewer, 1952a, b). There also appears to be significant differences among anurans in the amounts of neurohypophysial hormones present in the posterior pituitary. The toad (Bufo bufo) neurohypophysis contains far more hormonal material affecting water balance than does the frog Rana temporaria (Jorgensen, 1950c).

Quite variable results were found when examining the action of oxytocin on the ventral skin of Li. ewingi (102 mg/cm^2/hr - 185 mg/cm^2/hr). However, McLanahan and Baldwin (1969) found large variability (135 - 1425 mg/cm^2/hr) in the uptake of water by the pelvic skin in intact toads. Several other factors could have affected the variability of the present results.

The ground glass surface at the end of the flared tubes, resulted in considerable differences between the inside and outside diameters and thus in surface area (0.57 cm^2 and 0.95 cm^2 respectively).

In all cases the skin was attached firmly, but not too tightly over the flared tube and as excessive stretching may have caused damage. The acute angle at the outer

most edge of the flare may have prevented the skin from lying flat across the plane surface, particularly where the skin was thicker.

After electrical measurements were completed, the tube walls were mopped dry inside (above the level of the contained Ringer) and outside. The act of inserting fairly tightly rolled tissue into the tubes to mop up water droplets may have caused the skin diaphragm to bulge out (like the action of the finger testing the seal). This could have loosened the skin diaphragm stretched across the tube or at least allowed a liquid film to exist between the skin and the glass, and in contact with the Ringers solution inside the tube. When placed in Ringers solution or that containing neurohypophysial hormones, water would have been drawn through a larger area of skin in such tubes, thereby increasing weight loss from the tubes, thereby increasing weight loss from the tubes. A second, perhaps more important factor is the discovery by McLanahan and Baldwin (1969) that the ventral pectoral integument of the toad Bufo punctatus took up insignificant amounts of water, while that of the ventral pelvic integument took up water at a very high rate. More relevant are the similar findings by Bentley and Main (1972) for the arboreal frog Hyla moorei.

The specimens of Li. ewingi and Le.hochstetteri were so small however, that more concern was felt about getting as large an area of skin as possible (and more measureable weight changes) for permeability measurements than measuring the permeabilities of pectoral and pelvic ventral areas. This would have made little difference in Le. hochstetteri where it appears that the skin is uniformly permeable, or in L. aurea which were big enough to allow the exclusive use of the skin from the posterior abdomen for permeability measurements. This is reflected in the appropriate figures for these two frogs. However, in Li. ewingi where the diaphragm preparations invariably included most of the ventral skin, varying proportions of

less permeable pectoral skin could create widely differing results. It is probable that ventral skin permeability differences exist in Li. ewingi and it would not be unreasonable to expect them in L. aurea. The ventral (pelvic) permeability in Li. ewingi may therefore be higher than the present mean figure suggests.

While the concentrations of the hormones may not be in the physiological range normally present in the frogs, and oxytocin and vasopressin may not actually be present, the close structural similarity between oxytocin and vasotocin has been mentioned and the permeability responses may not be too artificial. One Li. ewingi when caught from its position on the wall of the housing container was noticed to have a slightly reddened ventral surface. The dorsal diaphragm preparation was observed to have fairly normal values (refer to table for range of normal values) for dorsal potential (25 mv) short circuit current (11.0 μ amperes/cm^2) and permeability (5.1 mg/cm^2/hr), the ventral potential was elevated above normal (74 mv) the ventral short circuit current was increased (42.6 μ amperes/cm^2) and the ventral permeability considerably above normal (78.9 mg/cm^2/hr). As the bladder of this frog was noticed to contain urine, the hormonal effects may have in fact been declining.

Bentley and Main (1972) found that the integument appeared to be uniformly permeable in Xenopus laevis and Neobatrachus pelobatoides which, due to their natural aquatic or fossorial habits, are most symmetrically exposed to moisture. They also found that in other species like the tree frogs though, (Hyla moorei) which normally only come into contact with water through their pelvic and abdominal regions, the abdominal skin was more permeable to water, and more responsive to the effects of vasotocin than was the pectoral or dorsal skin.

Posterior pituitary extracts are thought to increase the net transport of water through a modification of pore size of the skin membrane (Deyrup, 1964; Davson, 1970) and through an electro-osmotic mechanism (Capraro and Garampi, 1954).

(d) Electrical properties of the frog's skin.

i Introduction

(a) Like many other biological membranes active transport also occurs across frog skin. A frog, living in fresh water (a medium hypoosmotic with respect to the concentration of its body fluids) tends to lose salts through its skin as well as to gain water. A mechanism is present, however, for opposing this ion loss by an active transport process that carries Na^+ ions against the concentration gradient from a low external concentration to a high internal concentration in the extracellular fluid and blood (Davson, 1970).

The mechanism for the active sodium transport is very effective. As shown by Krogh (1937, 1939) this ion may be accumulated by Rana esculenta from solutions as dilute as 10^{-5}M (0.01 mM). Later work demonstrated that the skin of this frog in vitro could transport Na^+ actively from such external solutions, while the chloride moved passively, due to the accumulation inside the skin of positive ions, creating a potential which would build up until it was sufficient to accelerate the negative ions Cl^- to give equal net quantities of Na^+ and Cl^- passing in unit time. Active accumulation of Na^+ ions across the skin (in vitro and in vivo) has been demonstrated in a number of anurans from diverse families, including the Ranidae, Bufonidae, Hylidae, Leptodactylidae and Pipidae, and has also been shown to occur across the integument of some urodeles. Active chloride transport has been demonstrated in several frogs in vivo however, but in only one frog in vitro (Bentley, 1971).

Greenwald (1971), studying sodium balance in the leopard frog (Rana pipiens), found that one sodium depleted frog was able to reduce the concentration of sodium in a bath around it from 0.013 to 0.007 mM. He also found that sodium uptake increased with increasing external sodium concentration in a system that was able to be

described in terms of Michaelis - type enzyme kinetics with unchanged sodium loss rates. When depleted of body sodium however, Greenwald's frogs increased their rate of sodium uptake and generally decreased their loss rate at all external sodium concentrations.

If amphibian skin is removed and placed between two identical solutions of frog Ringer a potential is recorded, the inside being positive with respect to the outside and the magnitude of the potential being correlated with the active transport of salt across the membrane (Davson, 1970).

To prove that the potential difference (P.D. in m.V.) was really due to the active transport of Na^+, Ussing and Zerahn reduced the P.D. to zero by applying a counter E.M.F. The current indicated on the milliameter, required to reduce the P.D. to zero, then represented the current generated by the skin and was equal to the net inward flux of Na^+ if the flow of current was due solely to active transport of Na^+.

If the Cl^- ions moved down a gradient of electrochemical potential, reducing the P.D. to zero would prevent any net flux of Cl^- ions if the Ringer on both sides of the skin was identical. By neutralizing each positive charge carried by the Na^+ transport with an electron from the external circuit, the current in this external circuit is an exact measure of the rate of transport of sodium.

This explanation was checked using ^{24}Na and ^{22}Na in the fluids bathing the skin. On the average, the Na^+ influx amounted to 105% of the current while the outflux represented only 5%. The net Na^+ flux (influx-outflux) was therefore exactly equal to the short circuit current (S.C.C.).

Most experimental models of the translocation of substances across epithelial membranes are directly or indirectly derived from a model proposed by Koefoed-Johnsen and Ussing (1958) to explain the transport of Na^+ across the frog skin. The epithelial cell of

the stratum germinativum is described as an asymmetrical system. The outward facing membrane is permeable to Na^+ and the inward-facing membrane permeable to K^+. A pump actively transports Na^+ from the cell (through the inward facing cell membrane) and brings in K^+ presumably by a $Na^+ - K^+$ linked mechanism. The idea then, is of an epithelial membrane, the outer facing membrane of which is highly permeable to Na^+ and moderately permeable to Cl^- but impermeable to K^+. The inside facing membrane must be highly permeable to K^+ and Cl^- and relatively impermeable to Na^+.

The exchange of K^+ for Na^+ keeps the cellular concentration of Na^+ low, thus establishing a Na^+ concentration gradient across the outer barrier. This gradient results in a net flux of Na^+ from the outside of the cell. The K^+ accumulated in the cell by the pumping mechanism can diffuse back towards the inside but not towards the outside since only the inner facing membrane is permeable to K^+. The result is a net transfer of Na^+ from the outside solution into the inside solution. Cereijido and Rotunno (1970) state that under physiological conditions, the pump is assumed to operate neutrally, exchanging one Na^+ for one K^+. Davson (1970) provides some evidence however, that the pump is not electrically neutral, and the Ussing model may be incorrect in this respect.

The relationships between ATP hydrolysis and the translocations of Na^+ and K^+ in erythrocytes has been studied by Sen and Post (1964) who estimated that the extrusion of 3 Na^+, coupled with the uptake of $2K^+$, costs the cell one high energy phosphate bond. These data confirmed an earlier study by Leaf and Renshaw (1957) who also arrived at a figure of one high energy phosphate consumed for every $3Na^+$ transported, based on the stimulation of Na^+ transport and oxygen consumption by isolated frog skin under the influence of neurohypophyseal hormones. The 3:2 ratio could be present here also.

Cereijido and Rotunno (1970) have introduced a new non-transcellular model for the transport and distribution of Na^+ to account for certain findings not explained by transcellular models. These transcellular models adequately describe Na^+ movement under short circuit conditions with identical Ringers solution on both sides of the skin. However, very seldom is the outer facing membrane in contact with a solution in which Na^+ is as concentrated as in frog Ringer. The essential problem was to explain how high concentrations of Na could exist in the epithelium at concentrating 100 times greater than the bathing solution. The transcellular models would predict that since Na^+ enters the cellular compartment by a passive mechanism, the concentration in the cell should be still lower than in the outer bathing solution. Rotunno *et al.* (1966) suggested that the problem could be solved if epithelial Na^+ were compartmentalized and only one fraction was involved in Na^+ transport across the epithelium. This has since been shown to be true by the same workers. The model proposes that Na ions go across the epithelium without entering the cytoplasm by travelling around the cell. This mechanism is essentially independent of the one used by the cell to maintain its Na balance.

Similar rates of water loss but quite different rates of water uptake for hydrated frogs from different habitats would suggest that membrane structure is also associated with a favoured direction of water flow (e.g. inwards).

Thorson (1955) indicates that a number of earlier workers reported experiments demonstrating that Amphibian skin has an inward permeability to water which is greater than its outward permeability but that other workers had found that water passed equally readily in both directions through frog skin. Cohen's (1952) study of Salamanders found just the opposite however. The aquatic *Triturus torosus* lost water 10 times faster than it gained

water, Aneides lugubris lost only 1.25 times faster than it gained and Ensatina eschscholtzii xanthoptica lost 4.66 times faster than it gained. The frog Chiromantis xerampelina however exhibits a very high rehydration rate and the lowest evaporative water loss ever measured in an amphibian. Dorsal skin permeability measurements have not been made in this species however (Christensen 1974). MacRobbie and Ussing (1961) using the rather elegant measurement of the osmotic behaviour of Rana temporaria abdominal skin epithelium by microscopic means concluded that the outward facing boundary of the epithelium was much less permeable to water than the inward facing one and that the application of ADH to the inside bathing solution increased the water permeability of the outward facing boundary, whereas the inward facing membrane was unaffected. Unfortunately, no measurements were made on dorsal skin where major evaporative water losses could be expected in the living frog. Following this Whittembury (1962) estimated the equivalent pore radius at the outer and inner face of the skin epithelium. An equivalent pore radius of the 4.5A for the outer surface and one of 7A for the inner surface were obtained. The effect of ADH when added to the inner surface was to increase the outer 4.5A pores to about 6.5A.

Peachey and Rasmussen (1961) have done similar work to MacRobbie and Ussing (1961), working with the urinary bladder of the toad Bufo marinus. Their in vitro experiments showed that the serosal surfaces of the epithelial cells were freely permeable to water while the mucosal surfaces were normally relatively impermeable but became permeable when the serosal surface of the bladder was treated with neurohypophysial hormones. Bentley, (1961) also found directional differences in the permeability to water of the isolated urinary bladder of the toad B. marinus. Water transfer down an osmotic gradient was found to be 1.8 times more rapid towards the

serosal than towards the epithelial (mucosal) side. Vasopressin increased this difference so that water was moving 4.9 times as rapidly to the serosal side. Bentley also mentions that in frogs skin, in the absence of neurohypophysial extracts, there are differences in rate between inward and outward movement of water.

A comparatively early paper by Boyd and Whyte (1938) recognised that frogs placed in water and injected with pituitrin showed a considerable increase in weight due to augmented water flow. However, they also investigated the effect of injection of pituitrin on the loss of weight of frogs taken out of water. Boyd and Whyte found that under these conditions, pituitrin inhibited the normal loss of water from the body. The smallest dose of pituitrin which produced the maximum inhibition of water loss was about the same as that producing the maximum uptake of water by frogs. The time course of action was similar in both cases. Pitressin (vasopressin) and pitocin (oxytocin) were found to be equally effective in inhibiting water loss, but neither was as effective as pituitrin in this respect.

(b) The electrical properties of the frogs' skin in the presence of neurohypophysial peptides.

In addition to increasing the permeability of anuran skin to water, neurohypophysial peptides also promote active sodium uptake, (Bentley, 1971). This has been demonstrated in a number of frogs and toads, and also in some urodeles.

The effects of oxytocin and vasopressin on the P.D. and S.C.C. values of the frog's skin has been deliberately dealt with separately from the permeability effects, as the two processes are quite distinct (Bentley, 1971).

Bourguet and Maetz (1961) showed that the relative activities of different neurohypophysial peptides on water and sodium transfer were not parallel, and from

this, they have argued that these hormones have independent actions on the epithelial cell sites for active transport and water permeability. Hong's (1957) work with metabolic inhibitors, cold and anoxia which prevented mediation of H_2O uptake suggested cell mediation. Also, some urodeles and Xenopus laevis exhibit the natriferic (sodium) but not the hydroosmotic (water) response. "It is uncertain whether the natriferic effect of the peptides is of osmoregulatory significance, but may reflect some osmotic significance in the ancestors of this group" (Bentley, 1971).

Lin (1971) makes the interesting comment that the evolutionary emergence of the $Na^+ - K^+$ exchange transport process not only provided a way to control the specific concentrations of Na^+ and K^+ in the cytoplasm, but also, and more importantly, endowed the cell with a means of regulating its volume through adjustment of the internal osmotic pressure. This would free the cell from its dependence on a rigid wall structure for the prevention of osmotic lysis. Davson (1970) describes experiments where Ringers solution bathing the inner surface of the skin was diluted with water. The skin swelled, but very soon returned to its original volume, indicating a loss of osmotically active material, presumably KCl.

Sawyer (1951a) administered a mammalian neurohypophysial preparation (pituitrin) to Rana pipiens individuals and found that the rate of net inward movement of water increased from the control level of 4.54 ± 0.51 $\mu l/cm^2/hr$ to 26.7 ± 1.88 $\mu l/cm^2/hr$. Koefoed-Johnsen and Ussing (1953) concluded that the skin pore size was increased under these conditions and these findings are supported by a lowered skin resistence to the penetration of Na^+, Cl^- and certain small molecules, under these conditions (Deyrup, 1964). Davson (1971) also suggests that vasopressin acts by the opening up of relatively large skin pores. By increasing the permeability of the outward facing membrane to Na^+, more Na^+ would be able

to reach the active pumps and active transport of Na^+ across the skin would be thus increased. It is of interest that the effects of neurohypophysial hormones on water permeability are preserved in frog bladder preparations fixed by formaldehyde and glutaraldehyde (Jard et al. 1966).

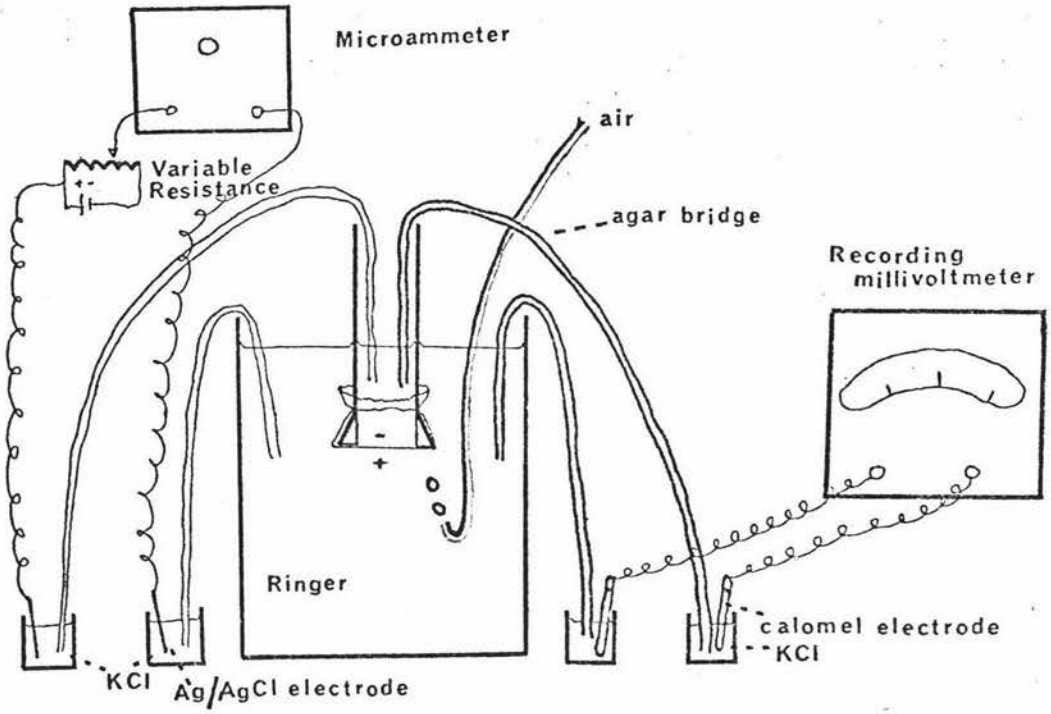
ii Materials and methods

The skin diaphragms were prepared as previously described. Normal strength Ringer was placed in the tube and in the beaker so that no chemical concentration gradients existed. Potential difference was measured with two matched calomel electrodes connected to either a Vibron Electrometer Model 336-2 or a Radiometer pH meter 28. The electrodes sat in phials containing saturated KCl solution and final contact with the solutions inside and outside the skin was made through saturated KCl-Agar bridges (refer to figure 12). These bridges were constructed by sucking hot saturated KCl in 3% Agar into polythene cannula tubing which was cut into suitable lengths when the Agar had solidified, and they were then stored in saturated KCl solution. They were repeatedly rinsed with distilled water before use. The KCl-Agar bridges are necessary because electrode potentials may be set up when measuring potential differences. If two wires are dipped into the solutions bathing the membrane, a potential difference will be observed between the wires but it will be erratic and mask the actual potential between the two solutions because either the wire will liberate ions into solution or the solution will deposit ions onto the metal. These electrode potentials depend on local conditions round the electrodes and can be overcome by using calomel and silver-silver chloride electrodes which operate on a similar basis to each other. The latter electrodes were prepared by electro chemically plating silver wire using a platinum cathode and N/10 HCl.

Figure 12

Apparatus for measurement of
Frog Skin Potential (m.V.) and
Short Circuit Current (μ.A./c.m^2).

Note: Outer skin surface faces into weighing tube, and inner skin surface, facing into the beaker, is the aerated one.


Microammeter
air
Variable
Resistance
agar bridge
Recording
millivoltmeter
Ringer
calomel electrode
KCl
KCl
Ag/AgCl electrode

A current of 2-3 milliamps was passed for 1-2 hours to effect deposition of AgCl. When the covering of AgCl was damaged or used up, or mainly after each experiment, it was redeposited and the electrodes stored in distilled water.

One is still confronted with the problem of making electrical contact between the controlled conditions around the electrode and the solutions bathing the membrane. When two solutions of the same electrolyte are in contact, the more concentrated tends to diffuse into the more dilute and results in a potential difference (liquid junction potential or diffusion potential) between the solutions.

If liquid contact is made between the solutions bathing the membrane and the solutions bathing the electrodes, junction potentials will be formed and will be measured as part of the potential across the membrane. KCl-Agar bridges remove this difficulty as both K^+ and Cl^- diffuse with the same speed and do not contribute to a diffusion potential. The potential that might arise from any other ion that happens to be present is "swamped out" by the high concentration of KCl, and the agar prevents the mass flow of the whole KCl solution out of the tubing. In operation the ends of the bridges were trimmed periodically to prevent the formation of air pockets.

When steady P.D. readings had been obtained (usually within an hour) two further electrodes (Ag/AgCl) were connected through salt bridges to the solutions on either side of the membrane (refer to figure 12). By the application of a suitable current and the adjustment of the variable resistors the P.D. could be opposed and completely abolished. The current reading (in μ amperes)

was then the short circuit current.[22]

iii Results

Mean P.D. Values across the dorsal and ventral skin of L. aurea and Li. ewingi are different, dorsal values being higher than ventral ones, and this is generally borne out in the individual measurements, though the values were very variable.

Consideration of S.C.C. values in these two species shows that ventral values are approximately double those of the dorsal skin (refer to table 4).

Some mean difference is also evident between dorsal and ventral P.D. measurements in Le. hochstetteri though several of the individual results agree closely. Similar dorsal and ventral S.C.C. values are evident.

P.D. Values of the ventral skin of the Hylids rose markedly under the influence of the two neurohypophysial hormones, and ventral S.C.C. figures increased dramatically.

Both dorsal and ventral P.D's in Le. hochstetteri increased considerably under the influence of the neurohypophysial hormones. The low and similar S.C.C. values were also increased.

The dorsal skin in both Hylid's however, showed a decrease in P.D. and in S.C.C. under the influence of the influence of the neurohypophyseal peptides in most cases.

22 Davson (1970) states however, that during short circuiting, the transport of Na^+ must be greater than when the skin is on open circuit since the anion drag involved in transporting both cation and anion is abolished.
The neurohypophysial hormones used were oxytocin (Pitocin or alphahypophamine) and vasopressin (Pitressin) both by Park Davis & Co.

TABLE 4

Skin Potential (P.D.) in m.V. and short circuit current in μ amperes/cm^2 through dorsal and ventral skin of three species of frogs. (Potential positive on the inside of the skin with respect to the outside).
(a) Non-stimulated. (Frog Ringer solution on both sides of membrane.)
(b) In presence of oxytocin 10 mU/ml. (ox)
(c) In presence of vasopressin 10 mU/ml. (vp)

	Potential Difference (mv)						Short circuit current (μa/cm.^{2})					
	P.D.Dorsal	+ ox	+ vp	P.D.Ventral	+ ox	+ vp	S.C.C.Dorsal	+ ox	+ vp	S.C.C.Ventral	+ ox	+ vp
Litoria aurea												
1.	46			29			12.63			18.95		
2.	15			29			8.63			24.53		
3.	31			21			9.26			21.26		
4.	39			30			10.53			28.42		
5.	41			20			10.53			18.53		
6.	30	24		41	112		7.79	6.53		16.67	54.39	
7.	32	20		30	60		7.89	5.26		23.16	44.21	
8.	27		22	18		20	8.21		8.42	15.37		20.00
9.	19		21	11		60	5.26		9.47	8.00		41.26
Mean	31.1	22.0	21.5	25.4	86.0	40.0	8.97	5.90	8.95	19.65	49.30	30.63
S.E.	± 3.3	±0.94	± 0.5	± 2.6	±26.0	±20.0	±0.7	±0.64	±0.7	± 2.1	±5.1	±10.6
Litoria ewingi												
1.	50			34			11.37			24.74		
2.	54			25			13.68			21.05		
3.	65			25			12.63			23.37		
4.	60			15			8.42			21.89		
5.	45	33		20	55		9.05	7.89		17.47	62.11	
6.	15	12		32	35		9.26	7.40		26.6	31.6	
7.	48	31		20	35		12.11	9.68		25.79	42.63	
8.	20		12	10		20	12.84		9.47	14.21		22.32
9.	42		24	22		51	9.26		6.95	13.47		48.21
10.	38			10			8.42			27.72		
Mean	43.7	25.33	18.0	21.3	41.7	35.5	10.70	8.32	8.21	21.63	45.45	35.27
S.E.	±5.1	±6.7	±6.0	±2.6	± 6.7	±15.5	±0.64	±0.7	±1.3	±1.7	±8.7	±12.8
Leiopelma hochstetteri												
1.	18			15			1.58			1.58		
2.	10			12			3.16			3.79		
3.	41	45		29	79		5.47	7.16		5.47	11.79 *	
4.	45	62		12	71		5.05	7.58		2.95	10.11	
5.	20	35		20	39		2.11	3.16		2.11	4.42	
6.	33		65	22		38	3.58		5.26	3.16		4.32
Mean	27.8	47.33	65	18.33	63	38	3.49	5.97	5.26	3.18	7.27	4.32
S.E.	± 5.7	±7.9		±2.7	±8.5		±0.65	±1.4		±0.57	±2.9	

*Note - oxytocin results from Le. hochstetteri (3) taken after normal permeability and electrical data had been obtained.
Le. hochstetteri (4) gave a higher than normal ventral permeability reading in the presence of oxytocin (see previous table) and Le. hochstetteri (5) was then used to check this, and gave a result in agreement with Le. hochstetteri (3).

iv Discussion

Bentley (1972) has stated that Na^+ transport can vary considerably in amphibian membranes and reflects the animals general condition and state of Na depletion. This may explain, to some degree at least, the variation in results in frogs of the same species. Therefore, although no firm conclusions can be reached, certain general points can be made.

The high dorsal P.D. and low S.C.C. in the Hylid frogs and the opposite situation in the ventral skin may at first appear puzzling if the P.D. is thought due to the movement of Na^+ ions. However, as reported by Davson (1970) Koefoed-Johnsen, Levi and Ussing observed that the higher the P.D. across a given skin, the lower were the fluxes of Cl^-. It was pointed out that if the P.D. were really due to a primary active transport of Na^+, the Cl^- fluxes would represent a partial short circuiting of the P.D. A high Cl^- flux would mean a small tendency for the Na^+ and Cl^- ions to separate. As the P.D. depends on this tendency of the ions to separate, it would be small in this case. Alternatively, a low Cl^- flux would lead to a greater separation and a higher P.D. Taken to its extreme, if Na^+ and an accompanying anion were transported in electrically equivalent quantities, no electrical P.D. would be observed.

However, the effects of the neurohypophysial peptides on the dorsal P.D. and S.C.C. of L. aurea and Li. ewingi are puzzling in that both of these values are decreased in most cases. Bentley and Main (1972) found that in the absence of vasotocin, sodium transport (S.C.C.) was relatively uniformly distributed around the integument of Bufo marinus, Xenopus laevis, Rana pipiens, Neobatrachus pelobatoides though not in Hyla moorei. They theorized that relatively greater changes in osmotic and evaporative permeability may have caused corresponding changes in S.C.C. and Na permeability.

Bentley and Main further noted that though no significant increase in the osmotic permeability of the skin from the dorsal or pectoral areas was seen in Rana pipiens, vasotocin was observed to facilitate the S.C.C. in these areas. This apparent inconsistency was not seen in Hyla moorei, but is a well known characteristic of Xenopus skin (and most Urodeles) where neurohypophysial peptides increase Na^+ transport, but have no effect on osmotic permeability. What these workers do not explain however, is that while the dorsal P.D. decreased, and there was little effect on S.C.C. under the influence of vasotocin, dorsal skin permeability markedly decreased in H. moorei.

Under the influence of oxytocin and vasopressin, dorsal P.D. and S.C.C. were both observed to decline in the Hylid frogs used in the present study though vasopressin did have a positive effect in Li. aurea but no significant effect was noted on dorsal skin permeability to water.

The small number of Le. hochstetteri used prevents any definite conclusions from being drawn, but it appears that both dorsal and ventral skin have similar increases in P.D. and in S.C.C. The considerably lower S.C.C. values, both dorsal and ventral, in Le. hochstetteri, suggest a lower uptake of Na^+ in these frogs. However, Na^+ uptake appears to be greater through the ventral skin than the dorsal skin in both L. aurea and Li. ewingi, further supporting the idea of a specialized ventral integument.

A dual type of bladder membrane is discussed by Davson (1970) using the model of Lichenstein and Leaf (1965). The urinary surface of the mucosal cells is represented as a dual barrier, a dense diffusion and a porous barrier, in series. All substances including water are retarded at the diffusion barrier. Vasopressin enhances the permeability of this tissue to urea and Na^+ by an effect on the dense diffusion barrier, and to water by

an effect on the porous barrier.

The discovery of the adenyl cyclase system by Sutherland and Rall (1957) is of significance in this respect, as it constituted the first evidence for the existence of a receptor in a biological membrane. The finding that several physiological substances such as adrenalin and vasopressin affect cell metabolism by interacting with adenyl cyclase, resulting in the production of cyclic 3';5' A.M.P lends support to the theory that a hormone need not penetrate into the cell in order to produce a widespread and integrated response in that cell. Vasopressin has been found to influence cell permeability by regulating the concentration of cyclic -3', 5' A.M.P inside the cell. Orloff and Handler's (1962) work supports this concept by showing that cyclic A.M.P. mimics vasopressin in its action on the toad bladder, though Cuthbert and Painter (1968) do not consider it necessary to involve cyclic A.M.P in order to explain the effects of either ADH or theophylline.

Various workers have argued for the presence of one or two receptors. The likelihood of two receptors comes from studies of Heller, Bentley, Morel, Maetz and others who approached the problem from an evolutionary point of view, noting the well known natriferic effect of oxytocin in salamanders but no increase in water flow. An analogous situation exists in the fresh water fish Carassius auratus (Maetz 1963). More recently the actions of analogues of oxytocin and vasopressin on kidney tubules, the skin and urinary bladder of Rana esculenta have been examined in detail by Morel et al. (1969). The results are consistent with the view that the natriferic and water flow effects of the neurohypophysial hormones involve two separate and specific receptors.

This dual type of membrane, if present (completely or partially) in frog skin, could explain the different responses observed. The porous barrier, if present in the skin, may be a comparatively recent innovation in amphibia. A second possibility is the absence of suitable hormonal

receptor sites at this barrier. In either case, a lack of effect of neurohypophysial hormones here would account for the fact that water flux through the skin of primitive amphibians such as the Urodeles, Xenopus, and Le. hochstetteri is not increased. The natriferic effect of the hormones would be explained by the action on the dense diffusion barrier.

The action of oxytocin and vasopressin in causing a decrease in P.D. and S.C.C. in L. aurea and Li. ewingi is more difficult to explain. A partial explanation may be found in the opposing actions of calcium (Ca^{++}) and vasopressin. Ca^{++} decreases the net transport of Na^{+} across amphibian skin (Davson, 1970). Also, the presence of Ca^{++} ions within cell membranes is thought to increase the permeability to inorganic ions (for example chloride) also (Florey, 1966). Both of these actions would explain the P.D. and S.C.C. effects. Greater levels of Cl^{-} ions entering the cells would tend to reduce the P.D. towards zero as already explained, while a blocking action on Na^{+} transport would reduce the S.C.C., as would a blocking of passive Na^{+} entry at the outer skin membrane.

It is also possible that small permeability effects could be mediated by this means in that water movement by electro-osmosis would be partially or completely abolished via the action of Ca^{++} on Na^{+} transport. It may be relevant that all these effects were observed in dorsal skin of the Hylids, as Elkan (1968) has reported the presence in more terrestrial anurans of an acellular skin layer (G. layer). This layer was found mainly in the dorsal skin, and was associated with massive amounts of calcium.

It may be thought therefore that this dorsal depot of calcium could be associated with the P.D. and S.C.C. effects. However, Elkan's results show that the G. layer is absent in Li. ewingi and is present in L. aurea only in very scattered form. Possibly in these frogs, levels of cellular Ca^{++} are sufficient to explain the observed effects. The absence of this layer in Le. hochstetteri

may explain the elevation of P.D. and S.C.C. in both dorsal and ventral skin under the influence of oxytocin and vasopressin.

The lack of effect of these hormones on the dorsal skin permeability of L. aurea and Li. ewingi may be explained by further referring to work done with the toad bladder. As previously mentioned, it is possible to dissociate the actions of vasopressin on the two processes of active Na^+ transport and H_2O- permeability. In addition, Bentley (1959, 1960) showed that high concentrations of Ca^{++} inhibited the vasopressin induced rise in H_2O permeability, but not the increased active transport of Na^+. If this blocking effect on vasopressin could also be shown to occur in the dorsal frog skin, it would partially explain the lack of permeability effect. Petersen and Edelman (1964) found no Ca^{++} effect on the vasopressin induced rise in the permeability in other bladders. It appears that Ca^{++} can dissociate the effects of vasopressin on water and urea movement from its effect on Na^+ transport. The results also indicated that Na and urea movement were controlled at different sites, in disagreement with the work of Lichenstein and Leaf (1965). Herrera (1971) explains the discrepancy on the basis of the different geographic origins of the toads used in both studies. A unifying mechanism for the three frog species used in the present study may not therefore be possible. A further possibility is provided by Wright and Snart (1971) who have reported the existence of an inhibitor to the action of oxytocin or vasopressin (vasopressinase) on water transport across the toad bladder which causes a loss of sensitivity to the hormone. An inhibitor substance formed in the bathing medium during overnight incubation of toad bladders was found to completely inhibit the vasopressin stimulated water transport across fresh bladders, but did not affect the stimulated Na^+ transport. These workers have suggested that the enzyme vasopressinase acts by destruction of the hormone before it reaches the active

site. Regional skin concentration differences of vasopressinase could account for regional permeability differences under the influence of neurohypophysial peptides.

Bufo marinus and Rana pipiens, according to Elkan both have the G. layer, B. marinus very abundantly. However, neither of these species show the dorsal P.D. S.C.C. and permeability decrease (Bentley and Main 1972). Only traces of the layer are present in Xenopus laevis and it is absent in Le. hochstetteri. Both these frogs appear to show a fairly uniform P.D. and S.C.C. response dorsally and ventrally to neurohypophysial peptides, and a lack of permeability response. However, this may be due to the relatively unspecialized skin of these primitive amphibians previously suggested where the necessary receptors of mechanisms for hormonal effects on water permeability may not be present.

Differences in dorsal and ventral ion and water permeability may be more widespread in the Hylidae than Bentley and Main indicate.

(e) Skin Histology

i. Introduction

Elkan (1968) describes the layers normally found in anuran skin thus:- A thin stratum corneum of keratinized epithelium,
An epithelium, which may have a depth of from 3-15 cells,
A basal membrane.
The stratum spongiosum containing various glands in a network of collagen elastic and smooth muscle fibres, nerves and capillary blood vessels. It also contains a variety of chromatophores with variously coloured pigments.

The stratum compactum, consisting of a few or many layers of comparatively thick collagen fibres.
The tela subcutanea which forms the outer lining of the subcutaneous lymphatic space. It carries blood vessels, nervesand elastic fibres and sends centrifugal connecting pillars out through gaps in the stratum compactum to link up with the stratum spongiosum and the epithelium.

Noble (1954) regards the development of alveolar and in some cases tubular glands as the chief evolutionary advance shown by the integument of the Amphibia over that of the fish. Two types of alveolar glands are common to the three orders of amphibia; the mucous glands and the granular or poison glands. The secretion of the mucous glands serves as a lubricant in water and to keep the skin moist on land. The granular glands produce a secretion very harmful to the mucous membranes of the eye and mouth, and may cause nausea, a weakening of respiration, muscular paralysis and circulatory collapse in other vertebrates.

Species differ greatly in the virulence of their poison. Bufo marinus produces one of the most virulent poisons known among the amphibia which may kill dogs that have not learnt to leave the toad alone. The granular glands are often of large size and clustered in pads or ridges. The mucous glands are widely spread over the body, and never reach a large size (Noble 1954).

The number and distribution of the two types of glands were therefore noted in the three frog species under study, the reason being that more terrestrial frog species may be expected to require greater numbers of the mucous glands to keep the skin moist.

However, Elkan (1968) made the exciting rediscovery of an acellular ground layer (G.layer) in the skin of many anuran species. The layer is situated between the stratum

compactum and the stratum spongiosum and consists of acid mucopolysaccharides and calcium. Most earlier workers have not described this layer or realized its potential significance.

Elkan found that there was a gradual increase in G from the completely negative Ascaphidae, Leiopelmidae and Pipidae, towards the Ranidae and Microhylidae which were, without exception, positive. The increase in G was not a regular one and the anomalous presence or absence of the layer in some frogs tends to obscure the pattern to some extent. The overall impression gained was that appreciable amounts of G were to be found the higher on the evolutionary scale it was searched for, families low on the evolutionary ladder containing most of the totally or predominantly aquatic species being devoid of dermal G. The amount of G seemed to be more correlated with the ecology of any anuran than with its position on the evolutionary scale. Elkan also mentions the combination of calcium and proteins and the considerable water binding properties of mucoid substances which makes anuran G eminently suitable as a water regulator, slowing desiccation in terrestrial situations and storing water on re-immersion. This explains the prevalence of G in the dorsal skin, this region of the frog being subjected to greater dehydration than the more protected ventral skin.[23] Water uptake takes place primarily through the ventral pelvic skin however, and an impermeable barrier in this region would reduce the rate of rehydration after desiccation.

Elkan states it is no loner correct to say that the most valuable "invention" to assist amphibian

[23] Elkan's study unfortunately did not include Chiromantis xerampelina or Phyllomedusa sauvagii both of which were referred to previously as having very low rates of evaporative water loss.

survival would be a more impermeable integument. He claims that the invention has been made, and it only remains to perfect it and extend its use to all non-aquatic species.

The presence of G was searched for in the two introduced frog species where it might have been expected at least in Li. ewingi. One of Elkan's anomalous findings, however, was that Li. ewingi did not possess this layer and it is puzzling, in view of its postulated protective properties that the G layer was predominantly found in larger frogs. Elkan did find the layer, in fragmentary form in Li. aurea but not in Le. hochstetteri where, on evolutionary grounds it would not be expected.

Skin thickness was also measured from the different skin regions in the three species of amphibians used, as it appeared from handling the skin for permeability measurements, that the skin of Li. ewingi was thinner than in Le. hochstetteri of comparable size. The stiffness of the skin of Le. hochstetteri appeared more closely comparable to that of Li. aurea.

ii Whole skin mounts

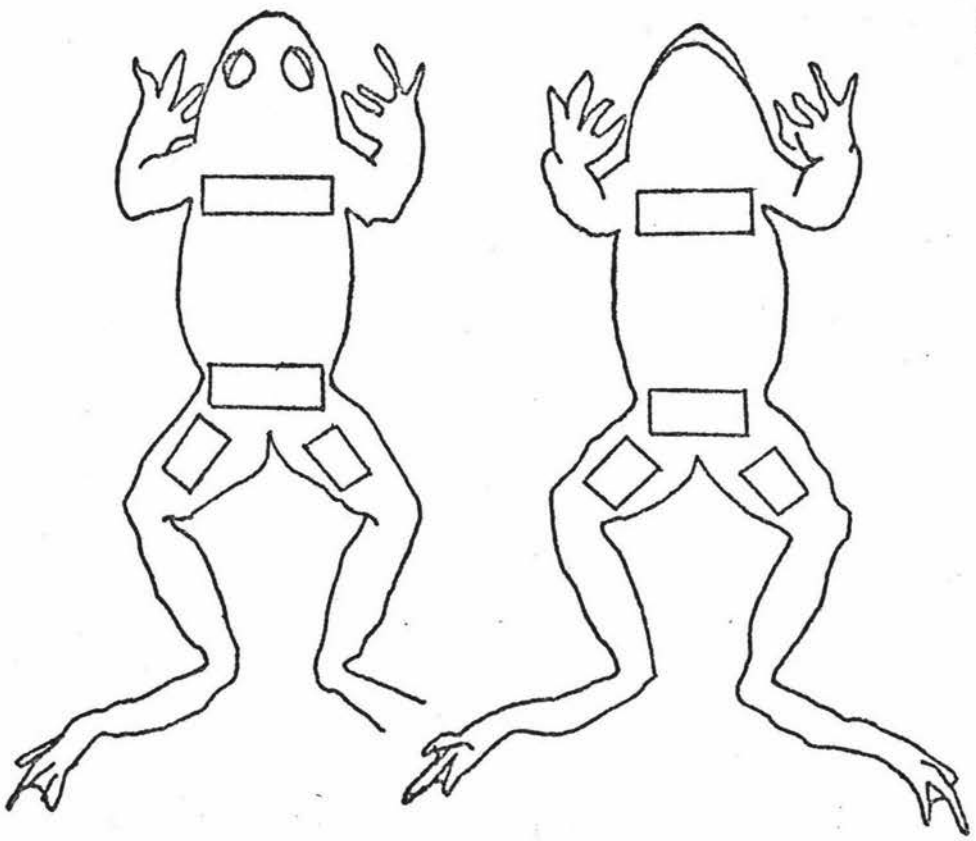
1. Materials and methods.

The method is essentially that of Czopek (1955). Skin sections from left and right sides of the body and different areas (refer to figure 13) were treated separately. Skin from one side was immersed in 3 per cent hydrogen peroxide for three days (bleach) after removal from Bouin's solution. The sections were then taken through 70 per cent - absolute alcohol, xylol and were mounted in Canada balsam. 1 mm^2 areas were counted for poison and mucous glands in each section, and the results were averaged.

The skin of the opposite side of the body in selected frogs was prepared for sectioning.

Figure 13

Frog, Dorsal and Ventral Aspects, showing regions where skin sections were removed.



DORSAL

VENTRAL

2. Results (refer to appendix 3)

There was a generally scattered distribution of mucous glands in the dorsal and ventral skins of the three frog species studied. Mean numbers of mucous glands always exceeded numbers of the larger granular poison glands, except in the dorsal posterior and dorsal hind-limb skin in Le. hochstetteri where aggregation of poison glands in the form of ridges or bulges comprised the majority of the glands present.

The numbers of mucous and poison glands were not very different in the skins of Li. aurea and Li. ewingi, though on a size basis, Li. ewingi would have had proportionately more. Dorsal aggregations of poison glands were seen in both these species, though mucous glands were invariably present in considerable numbers also. Lesser numbers of both gland types were seen in Le. hochstetteri, though the glands themselves were larger. Both dorsal and ventral skin of Le. hochstetteri appeared similar in gross appearance and in general distribution of skin glands (refer to Plate 4). However, the ventral skin of the other two species, with its "cobbled" appearance, was quite different from the relatively uniform dorsal skin in these two frogs. Examination of the whole-skin mounts showed that these varied areas consisted of aggregations of predominantly mucous glands, with clearer and therefore probably thinner channels devoid of glands separating these aggregations (refer to plates 5 and 6).

It also appeared that these "channels" were wider in the ventral pelvic region than in the pectoral area, and that they also occupied a greater area in the ventral skin of Li. ewingi particularly posteriorly .

Plate 4

Whole skin mount, Le. hochstetteri, ventral pelvic skin. Showing distribution of small, light coloured mucous glands (M) and larger, darker, poison glands (P) x80.

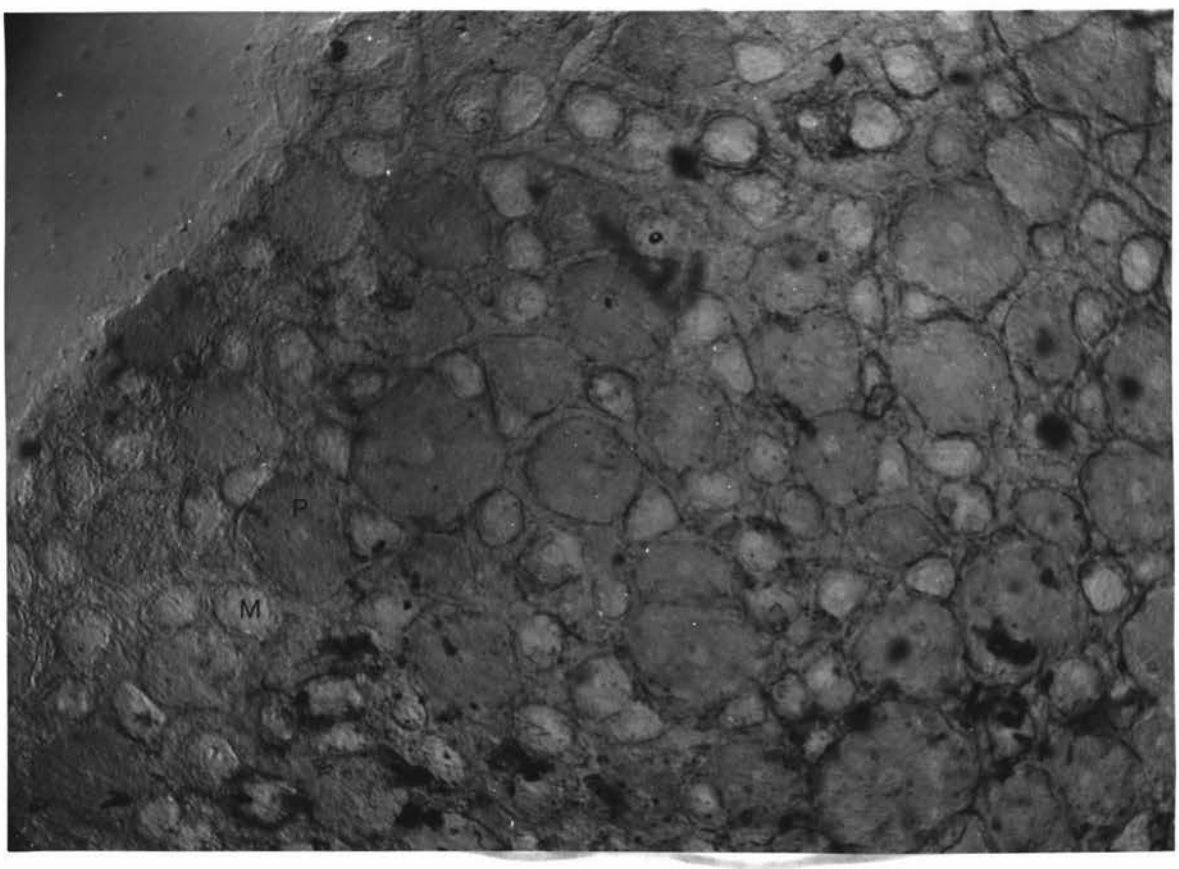
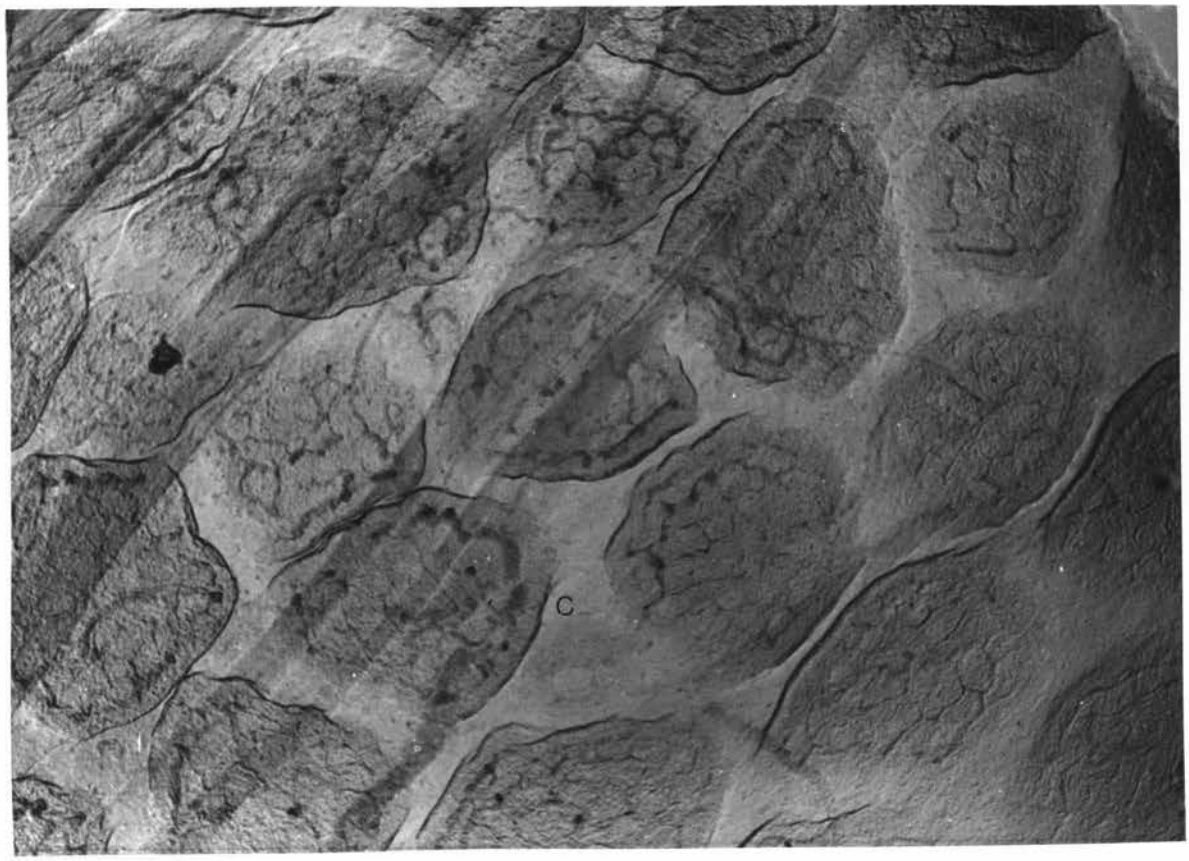

P
M

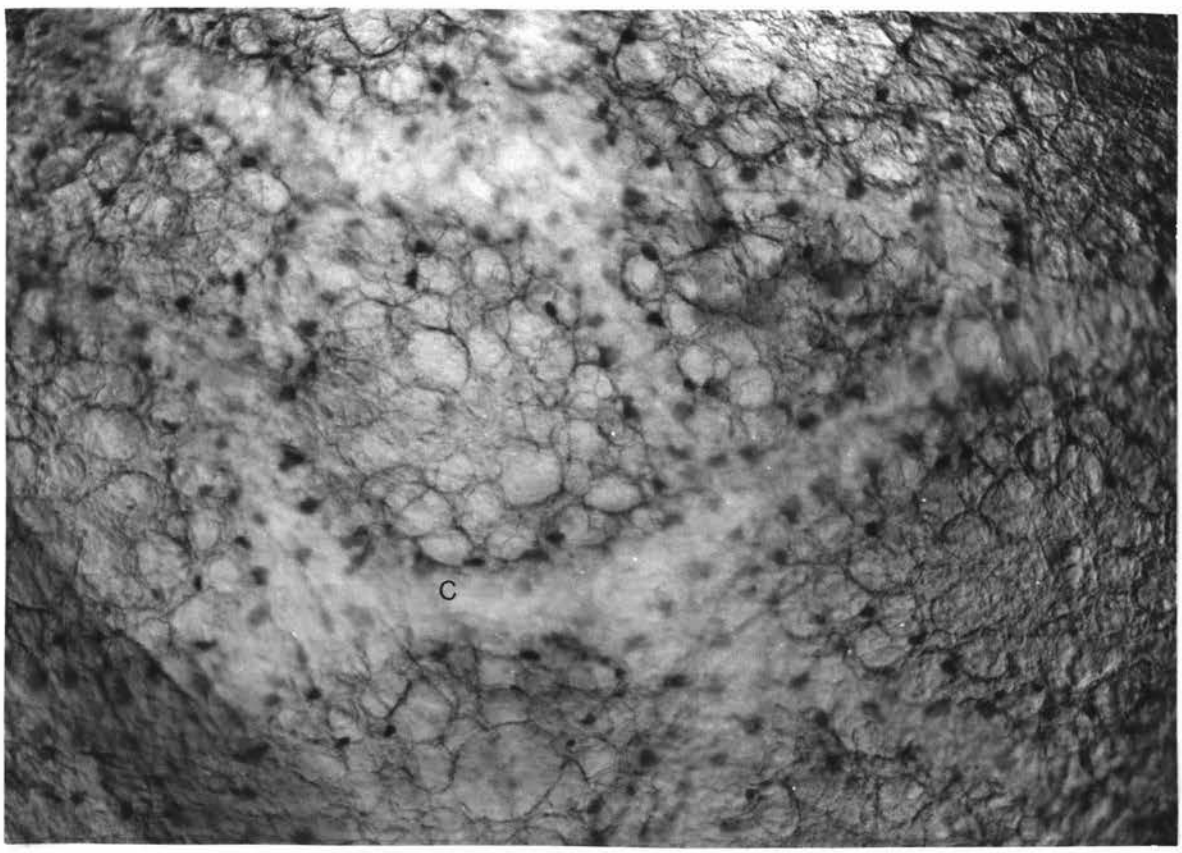
Plate 5

Whole skin mount, Li. ewingi, ventral pelvic skin. Raised groups of mucous glands separated by wide, lighter "channels" (C) x80.

Plate 6

Whole skin mount, Li. aurea, ventral pelvic skin. Raised groups of mucous glands separated by lighter "channels" (C) x80.


C


C

iii Skin Sections

1. Materials and methods.

Skin sections from the opposite side of the body than that used for whole skin mounts were embedded in paraffin. Sections were stained with Erlich's haematoxylin and counterstained with saturated eosin in 90 per cent alcohol.

The thickness of skin sections was measured using a graduated eyepiece calibrated against a stage micrometer.

2. Results. (refer to appendix 3)

Essentially, skin thickness in Le. hochstetteri (refer to plate 7) was similar to that in Li. aurea. The ventral integument (pectoral and pelvic) of Li. aurea did show thinner areas between much thicker regions containing mucous glands and an expanded epidermal layer (refer to plate 8). The skin of the ventral hind limb in these frogs, in addition to the thin areas, contained finger-like projections of blood vessels in the dermis extending up into the outer epidermal layer and containing capillaries at their extremities. These features were not seen in Le. hochstetteri (refer to plate 7).

Generally, the skin in Li. ewingi was thinner than in the other two species. However, the ventral pelvic skin and the ventral hind limb skin showed greatly thickened epidermal areas associated with mucous glands, alternating with very thin regions (down to 0.05 mm) (refer to plate 9) as seen to a lesser degree in Li. aurea. A more complex system of blood vessels was seen in the ventral hind limb skin of Li. ewingi. Large blood vessels were noticed under the dermis (in the tela subcutanea), many of which appeared to be situated beneath the thinner skin regions or "channels". Another series of smaller blood vessels was seen situated underneath the epidermal

Plate 7

Le. hochstetteri, ventral pelvic skin, L.S. Note relatively uniform skin thickness. Lighter mucous gland can be seen slightly to the left of centre and a larger darker granular (poison) gland, slightly to the right of centre. x500.

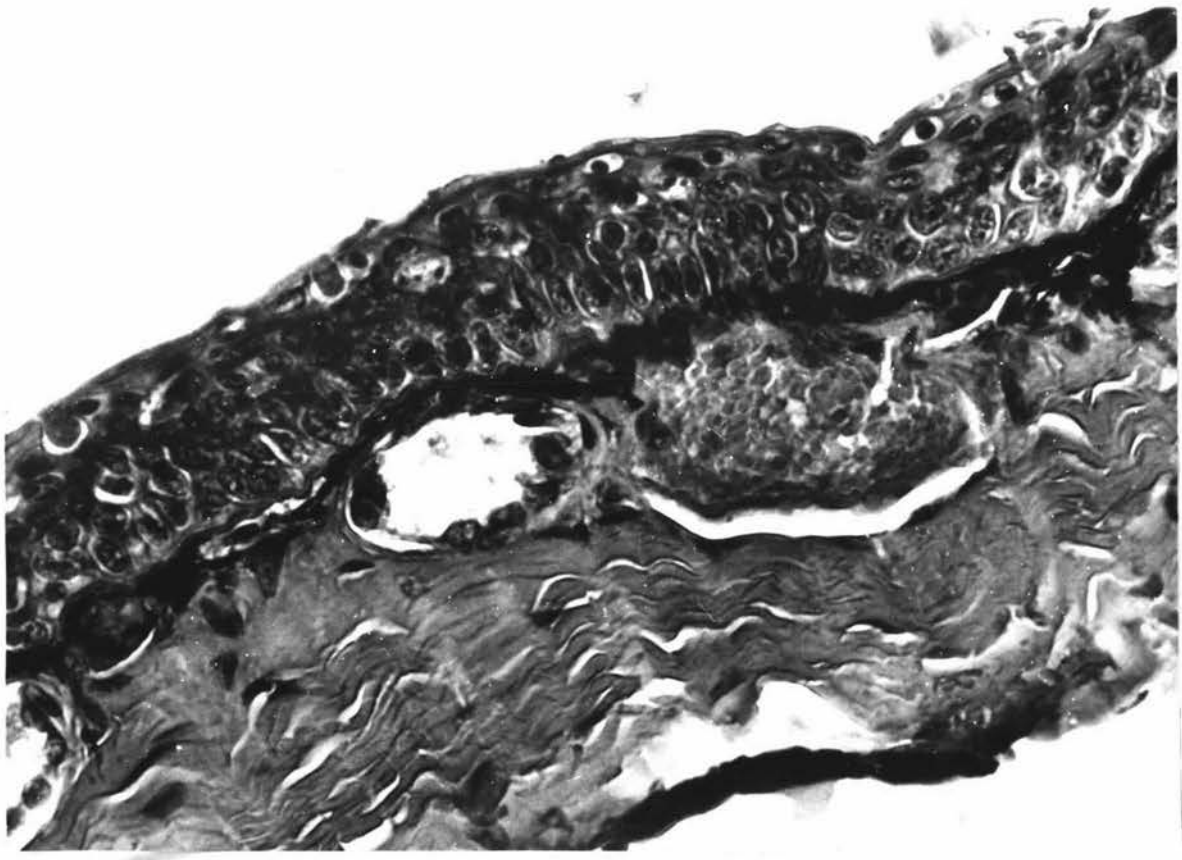
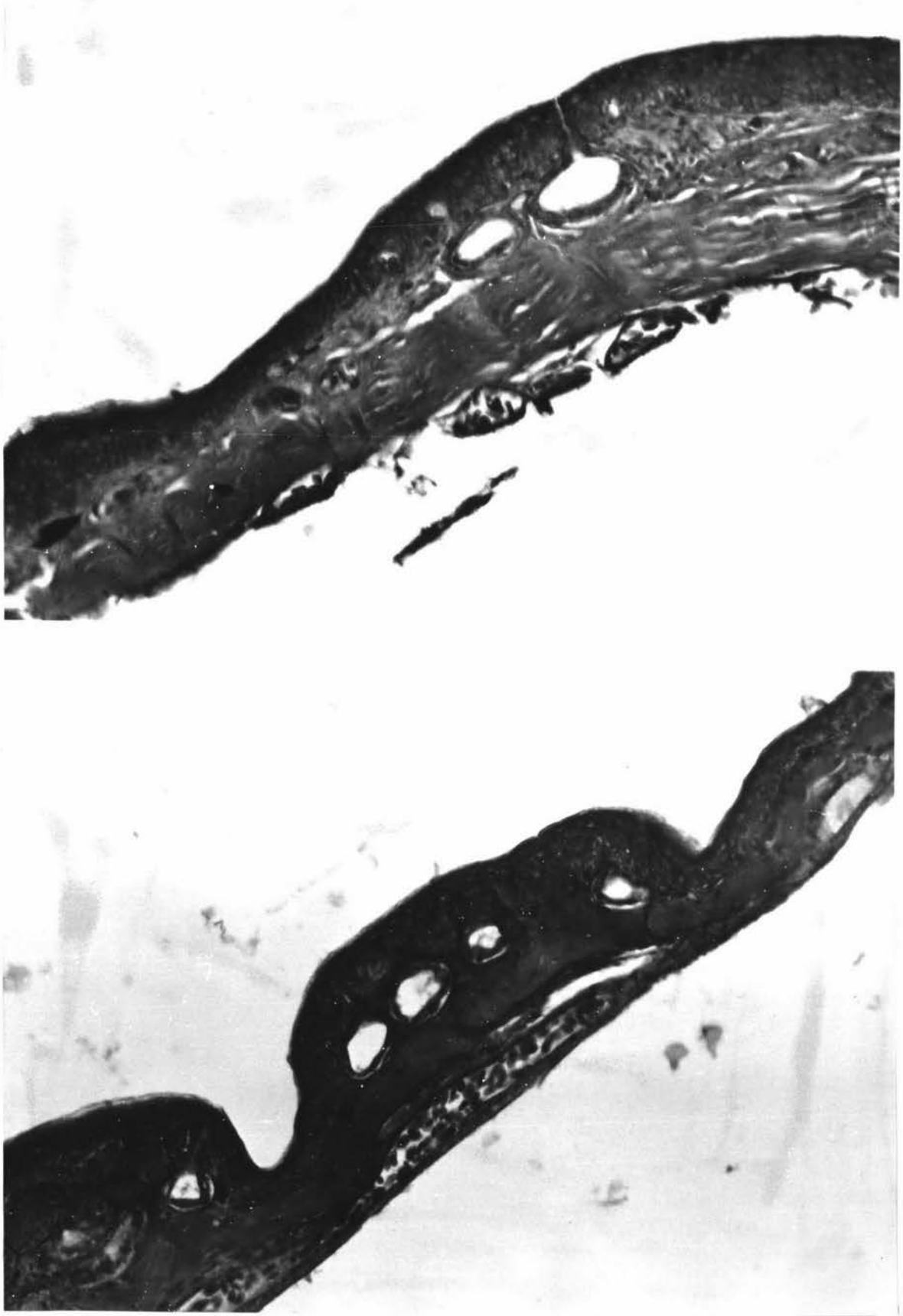


Plate 8

Li. aurea, ventral pelvic skin L.S. Note thicker skin areas containing whitish mucous glands. Thinner area present to left in plate. Blood vessels present. x240.

Plate 9

Li. ewingi, ventral pelvic skin, L.S. Thin skin areas visible. Note blood vessels also. x240.



layer (in the stratum spongiosum) and many vessels were also noted in the epidermis, extending in some cases into the outermost layers. This system of vessels was also noted in ventral pelvic skin of Li. ewingi but was not seen in this region in Li. aurea.

An acellular G layer was not seen in Li. ewingi or Le. hochstetteri but was seen in very scattered form in Li. aurea.

It was argued previously that Li. ewingi from the area of collection at Foxton at least, could be more terrestrial than those from Australia. Accordingly, it was thought that G may have been present in the dorsal skin of the Li. ewingi under study but this expectation was not fulfilled.

iv Discussion

Li. ewingi always looked considerably drier than Le. hochstetteri, and it was therefore surprising to find greater numbers of mucous glands in the dorsal skin of Li. ewingi. However, an explanation for this may be that the native frogs had in fact a layer of moisture on them but, in some cases, though these frogs had a shiny appearance, they felt dry. The whistling frogs, however, were slimy to handle in some cases.

The presence of a specialized ventral skin in the two Australian species is interesting. It was shown in a previous section, that the permeability of the ventral integument was greater in these two frogs than in Le. hochstetteri, and that this difference was heightened by the application of oxytocin and vasopressen. It was therefore thought that the presence of the lighter and therefore probably thinner channels between the aggregations of glands could provide a means of rapid water entry through the skin, if it is remembered that the rate of diffusion may depend partially on the thickness of the barrier being traversed. The lighter channels also appeared wider in the pelvic area of both

Australian frogs than in the pectoral region, and this may suggest structural differences which could result in permeability differences.

It is notable that the G layer is absent in urodeles and in anurans on lower evolutionary levels, and that in these amphibia there is no hormonal effect on permeability, only a sodium response. According to Ginetzinsky (1958) the increase in water permeability in epithelial structures in the presence of neurohypophysial hormones is due to an increased secretion of hyaluronidase or a similar mucolytic enzyme by the cells, the enzyme depolymerizing the mucopolysaccharides in the interstitial tissue and increasing in this way the intercellular permeability to water.[24] This hypothesis was later supported by the experiments of Ivanova and Natochin (1968) (reported by Kotyk and Janacek in 1970) who demonstrated that hyaluronidase at a suitable pH, corresponding to the type of enzyme used in the experiment, produces similar water permeability changes in the frog bladder to the neurohypophysial hormones. The changes were inhibited in the same way by higher concentration of Ca^{++} ions. This could explain the high levels of calcium together with mucopolysaccharides, as found by Elkan (1968). Presumably, water permeability changes are blocked by the calcium. There was also a marked lack of positive effect on the dorsal skin permeability or on P.D. and S.C.C. in the Australian frogs, which would suggest the mediation of calcium. This apparent paradox (i.e. the suggested mediation of Ca, but the absence of the G layer) is probably artificially created though. Curran *et al.* 1962/63,

[24] There is some evidence that Ca^{++} may be exerting its membrane effects by acting at the zona occludens or "tight junction" between cells (Lowenstein *et al.* 1967).

Herrera *et al.* (1963) found that addition of Ca^{++} to the solution bathing the outside of a frog's skin caused a decrease in net Na^{+} transport, while ADH added to the inner solution, caused an increase. The primary effect of both agents was to alter the Na^{+} permeability of the outer membrane and the size of the Na pool in the cells. It must be remembered, however, that a frog would not be normally expected to have a solution of the same concentration on both sides of its skin. Ussing's theoretical model was modified by Cereijido and Rotunno on the assumption that external Na^{+} levels would not have been as high as those used by Ussing. Similarly, levels of Ca^{++} would not be expected to be as high in pond water as in frog Ringer. More importantly, the dorsal surface may not be normally in contact with moisture, particularly in *Li. ewingi*. Even though during permeability studies the outer skin surface was bathed with 1/10 Ringer containing therefore a much lower concentration of Ca^{++}, it had previously been bathed with full strength Ringer for electrical measurements. Rubin (1962), discussing alkaline earth chelation,[25] states that the effect of the conbination on the nature of the metal ion and the ligand molecule, has interesting inplications for transport of the alkaline earths. The decreased charge, decreased hydration, and increased lipotropic character of some alkaline earth chelates may permit the metal ion in this form to approach the charged membrane and become available for transport. A change in the Ca^{++} ion level in the medium by secretion of hydrogen ion or calcium binding

[25] The term chelate refers to coordinating atoms linked in single ligand or binding molecule so that the combination with the metal ion results in a ring structure. The usual donor atoms of ligand molecules suitable for chelate formation with alkaline earths are oxygen, nitrogen and sulphur.

metabolite may alter the permeability of the membrane to other ions (Rubin 1962). This has been indicated by Rubin for the effect of calcium ions on the permeability of frog skin epithelium to Na^+ and Cl^- which may be due to several effects. These include, the selective covering or uncovering of carboxyl, amino or hydroxyl groups in the membrane, changes in the physical characteristics of the membrane protein by combination with calcium resulting in changes in the equivalent pore radius, or a direct action on metabolic processes and energy metabolism. Therefore, even if the dorsal skin outer surface was flushed with dilute Ringer, and this solution was used for permeability studies, the prior use of full strength Ringer may have caused membrane protein changes or pore blockage not immediately reversed by a more dilute solution.

It was observed in several of the *in vitro* skin diaphragm preparations that during the measurement of the S.C.C. the Ringer became rather frothy. It is known that mucous discharge occurs in response to sympathetic nervous stimulation (Lillywhite, 1971) and presumably, electrical stimulation mimicked this effect. Imamura *et al.* (1965) have shown that in at least one frog, the skin glands contain calcium ions and one could imagine that on discharge of mucous, the contained calcium could exert the effect noted by Curran *et al.* (1962,1963). Similar results could be envisaged in the living animal where, in some frogs at least, mucous is discharged during dehydration (Lillywhite, 1971). However this theory is not proved or disproved by the findings of Lillywhite working with *Rana catesbeiana* which possesses Elkans G layer. He found that frogs which frequently discharged mucous maintained steady states of evaporative water loss comparable to that of a free water surface. Frogs in which mucous gland activity was inhibited by sympathetic blockade demonstrated drying of the integument and declining rates of evaporative water loss.

These findings imply though, that the skin barrier is a more effective barrier to water loss than has been previously thought. Lillywhite's work does not show whether or not skin permeability is altered by mucous discharge.

The finding of epidermal blood capillaries and thin areas in ventral skin sections of Li. aurea and Li. ewingi could be important. The diffusion distance would be markedly reduced where these modifications are present. The reddened ventral skin observed in one Li. ewingi which was shown in a previous section to have elevated rates of water uptake through the ventral integument, may indicate an alternative and shorter pathway for water uptake. Water passing inwards through the ventral integument of hydrated species of Li. aurea and Li. ewingi may pass through the thinner ventral skin regions and into the lymph sacs, accounting for the greater ventral permeability in these frogs than in Le. hochstetteri. This rate of uptake could be augmented during dehydration by an effect of the neurohypophysial hormones on the permeability of the outer epidermal layers allowing greaterpassage of water through these layers and into the circulatory vessels. The possibility also exists that under these conditions, blood is "shunted" into the ventral blood capillary network to rapidly carry away the absorbed water.

The generally thicker skin in Le. hochstetteri and Li. aurea confirms the impression gained when handling the isolated skin diaphragms that the skin of Li. ewingi was thinner than in the other two species. This may explain why both dorsal and ventral skin permeability in Li. ewingi is greater than in the other two species. Cohen (1952) studying some Californian Salamanders, found that the aquatic Triturus torosus had a relatively unvascularized skin, relatively few poison or mucous glands, and a thickened epidermal layer. The two terrestrial plethodontids Ensatina escholtzii xanthoptica (a ground dweller) and Aneides lugubris of semi arboreal

habits had skins that were essentially alike morphologically, both being thin, highly vascularized and profusely supplied with mucous and poison glands. The greater ventral permeability in Li. aurea than in Le. hochstetteri may be a reflection of its specialized structure. The low overall permeability of Le. hochstetteri may be explained by the findings of Schmid and Barden (1965). Significant differences in permeability to water and in lipid content of the skin were observed among three species of anuran amphibians. These differences were correlated with differences in habitat preference. The skin of the aquatic mink frog Rana septentrionalis was less permeable to water and had a higher lipid content than that of Bufo hemiophrys, a terrestrial toad. The skin of the semi-aquatic tree frog Hyla versicolor was intermediate to these. A selective advantage of increased permeability to water was suggested by Jorgensen (1950) as an increased potential for water replacement. As previously stated, increased rates of water movement through amphibian skin under the influence of neurohypophysial extracts, appear to be related to increased rates of active Na transport and increased pore size. MacRobbie and Ussing (1961) found that the increase in permeability to water was localized at the relatively impermeable outer cornified layers of the epithelium. Jorgensen (1950) found that the effect of neurohypophysial extracts was greater in terrestrial species than in aquatic species. Schmid and Barden's argument, therefore, is that the difference in response of species from different habitats might be explained by the presence of more than one barrier to water flux. If the lipid content of the skin has an independent effect on water flux, both lower permeability to water and a lower response to neurohypophysial extracts would be expected of aquatic species. Conversely, terrestrial species, with lower skin lipid and a greater proportion of the water barrier responsive to neurohypophysial extracts, would have increased permeability

and increased hormonal control of water uptake. This statement makes no allowance for differences in dorsal and ventral skin lipid content. Schmid and Barden (1965) found that leg skin bags exhibiting lower water permeability had higher lipid levels and vice-versa. If therefore, the results presented here show a lower dorsal than ventral permeability to water, it would be reasonable to expect a higher dorsal lipid content. This could then result in a lack of hormonal control of water uptake. It may also mean that amphibia with low and fairly uniform skin permeability, such as Xenopus laevis and Leiopelma hochstetteri, have relatively high cutaneous levels of lipids. The lack of hydo-osmotic response can then be explained, using Schmid and Bardens argument.

Schmid and Barden used leg bags to measure skin permeability and so an average value for dorsal and ventral skin would have been obtained. Although lipid analysis was carried out on separate dorsal and ventral skin, these determinations were pooled. Unfortunately, no figures for the distribution of lipid in the dorsal or ventral skin separately are given but the layer appears to have a distribution opposite to that of Elkan's (1968) G layer, in that more lipid is present in more aquatic species, apparently to decrease skin permeability. This would solve the problem, partially at least, of body fluid dilution in water-living frogs.

Cereijido and Rotunno (1970) discuss the existence of pores in lipid membranes to account for the fact that small molecules cross cell membranes faster than expected on the basis of their solubility in lipids. The pores described may be standing or transient holes of any shape, follow a straight or tortuous path across the membrane, and their walls may have charges or groups with affinity for certain substances. Danielli (1954) proposed pores lined by the protein envelope of the membrane. These pores may have electric charges fixed to their walls.

Pores with such charges play a major role in explaining ion translocation and electrical phenomena (Cereijido and Rotunno 1970). Lipid soluble forms of even the more highly ionic alkali metals have been discussed (Rubin 1963). The postulate of Ca^{++} adhesion to a binding site in the cellular membrane layer has been invoked to provide an explanation for the observations on the effects of Ca^{++} on the permeability of membranes to the passage of other ions such as Na^{+} and Cl^{-} ions in the frog skin epithelium. The postulate has also been used to explain the rather specific effects of Ca^{++} in protecting the red cells of many fish from haemolysis, and its effects on the pore size of kidney cells of Necturus (Whittembury et al. 1960). Whittembury inferred that the Calcium content of the membrane exercises a delicate control over the equivalent pore radius and his results suggested that the action of vasopressin on membrane permeability may be due to an interaction with membrane calcium. The fact that hormones may interact with a receptor in or on a cell surface, producing a conformational change in the membrane protein and lipid has also been stated by Bittar (1971). The importance of calcium ions in the lipid barrier proposed by Schmid and Barden (1965) could therefore be considerable.

SUMMARY

1. Experiments with Li. ewingi and Le. hochstetteri show no detectable correlation between habitat and rates of water loss. Individuals of both species of comparable size exhibit similar rates of dehydration under the experimental conditions used.

2. Both Li. ewingi and Le. hochstetteri were able to withstand at least a 10 per cent loss of body weight as water (in the absence of bladder water) with no apparent ill effects.

3. Rates of evaporative water loss at higher humidities and lower temperatures were such that both species could spend considerable periods away from water under these conditions. These periods would be further increased by the presence of bladder water under normal conditions. An explanation is therefore provided for the finding of Le. hochstetteri some distance from water, especially where humidity is high, but also in apparently dry conditions. A partial explanation is provided for the finding of Li. ewingi in a terrestrial and partially arboreal habitat.

4. The generally secretive habits of Le. hochstetteri and the discovery of an individual in a small damp tunnel in a tree branch are thought to be important factors in the water economy of this species.

5. Significantly greater numbers of Le. hochstetteri may be found above ground level at night than during the day if climbing habits in captivity reflect normal activities.

6. Rates of water uptake in hydrated frogs, and after dehydration appear to be correlated with habitat in the species studied. Water uptake in hydrated Li. ewingi was higher than in both hydrated and dehydrated Le. hochstetteri. The differences in water uptake rates in hydrated frogs were found to be due to at least two

factors - plasma osmolality and skin permeability. Water uptake rates through isolated skin were higher in more the terrestrial frogs, Li. ewingi than in the aquatic Le. hochstetteri and a third species tested Li. aurea.

7. Zonal differences were seen in the permeability of the skin of Li. ewingi and Li. aurea, ventral and probably ventral pelvic skin being more permeable to water than dorsal skin. Relatively uniform dorsal and ventral skin permeability was observed in Le. hochstetteri. These differences are thought to be of adaptive significance and to be at least partially due to structural differences in the skin of the different species.

It is proposed that these differences confer an independence from ponds and pools for the moisture requirements of Li. ewingi and that these frogs are able to satisfy their requirements by absorbing rain water and dew from damp vegetation.

8. A dramatic increase in rates of water uptake after dehydration was observed in Li. ewingi but not in Le. hochstetteri dehydrated to the same extent. In view of the reported involvements of neurohypophysial hormones in intact amphibia and supported by in vitro experiments with oxytocin and vasopressin (A.D.H.) using skin diaphragm preparations, it is felt that the increase in water uptake is likely to be due to the effects of vasotocin in stimulating increased water inflow through the ventral skin of Li. ewingi but having little or no effect on Le. hochstetteri skin.

9. The significant hormonal responses of the isolated ventral skin of Li. aurea suggests the potential for a more terrestrial existence than pond life for these frogs.

10. The primitive nature of Le. hochstetteri has been discussed by various workers mainly in relation to anatomical and developmental studies. Further confirmation is provided by the physiological experiments

indicating a lack of hormonal effect on water uptake through dorsal or ventral skin in Le. hochstetteri. An effect was noted on Na^+ uptake rates as reflected by the short circuit current preparation. Similar results have neen obtained by other workers with Urodeles and Xenopus laevis.

11. It was suggested by an earlier worker that the New Zealand native frogs are very susceptible to drying out. Evidence has been presented that at least one other frog species of comparable size loses water at similar rates. However, this species (Li. ewingi) can take up water rapidly when hydrated through the specialized ventral integument and at considerably greater rates when dehydrated. These advantages are thought to be of major importance in enabling a terrestrial and at least partially arboreal existence for these frogs. Le. hochstetteri appears unable to take up water at rapid rates even, most importantly, when dehydrated. This more than anything else, may limit this species to damp, mist shrouded hills or to wet areas near streams. Under these conditions where humidity is high, the low rates of water uptake may balance the low rates of evaporative water loss.

12. The primitive nature of the other two species of New Zealand native frogs makes it unlikely that their water exchange mechanisms would be very different from those described for Le. hochstetteri even though they are thought to live in drier areas. The fact that Le. hochstetteri and Le. archeyi were found together in one area in Coromandel suggests that differences between these two species may be slight, if the idea that more terrestrial amphibians exhibit a lower tolerance to prolonged hydration is supported.

Note Added In Final Typing.

Hall & O'Regan (1974) have very recently reported that Prostoglandin E_1 and Vasopressin stimulation of sodium transport in frog skin is consistent with the mediation of cAMP. Also in skins treated with Prostoglandin E_1 the short circuit current decreased after the application of indomethacin. This effect was attributed to a blocking of endogenous prostoglandin production in the skin.

Support for the idea of blood vessel specialization in areas of rapid water uptake, is provided by the work of Christensen (1974). He has recently found that in B. bufo, R. arvalis and R. esculenta, the mean skin capillary diameters were largest in the pelvic region and that, taking into account the high number and large diameters of the arteries and arterioles, the resistance to flow in the pelvic vascular system must be several times lower than in other parts of the skin. More interestingly, Christensen found that blood flow through the pelvic skin was regulated according to water uptake. In normally hydrated animals, the capillary blood flow was approximately the same in different skin regions (i.e. pelvic, belly, back, pectoral) but during the last phase of dehydration (maximum 19-22%) the blood vessels in the pelvic region were dilated, which, combined with a stagnation or pooling of blood, gave the pelvic area a reddish appearance. If the animals were placed on a moist substrate, the flow increased.

Of similar relevance to the present work is Christensens finding that the higher rehydration rate in Bufo bufo than in Ranidae was explained by the presence of Tube-like grooves on the surface of the skin acting to promote capillarity. The skin channels in Li. aurea and Li. ewingi could function in the same way, as Christensen found that frog skin (R. esculenta, R. arvalis, R. temporaria but not Xenopus laevis) was mostly smooth except for the highly permeable pelvic area which had a

rough surface showing some capillarity. This would have the effect of spreading a small amount of water over a larger area for rapid absorption.

Bentley and Main's (1972) work showing regional permeability differences in the skins of some amphibians is further extended by the work of Baldwin (1974) using the toads Bufo puctatus and Bufo boreas, and it has been found in these species that arginine vasopressin increased the net transfer of water across the ventral pelvic integument, but not the ventral pectoral integument. Adenosine 3', 5' - monophosphate (cAMP) increased the net transfer of water in the ventral pelvic integument of B. puctatus.

Schmid and Barden (1965) have indicated the relationship between levels of skin lipids in frog skin, and the permeability of the skin to water. A new paper by Watlington et al. (1974) hints at further involvement of epidermal lipids in regulatory mechanisms involving ions. Significant relationships had previously been found by these workers between Na^+ and Cl^- transport and whole skin lipid levels. In individual skins, the magnitude of the net Na transport and partial Na conductance were found to inversely or negatively correlate with cholesterol content and lysolecithin content and preportion. Also, a positive correlation was demonstrated between the magnitude of chloride flux, and hence chloride conductance, and the proportion of phosphatidylethanolamine in the skin. The present paper investigates epidermal lipids on the basis of the site of the sodium pump and attempts to assess the effect on epidermal lipids of high salinity environment (i.e. a lipid effect was suspected in the mechanism whereby pre-exposure or conditioning of frogs to a medium high in NaCl produces, in the isolated skin subsequently removed, a decrease in net Na^+ transport.

Significant lysolecithin - Na conductance and phosphatidylethanolamine - Cl conductance correlations were detected in the epidermis of unconditioned frogs

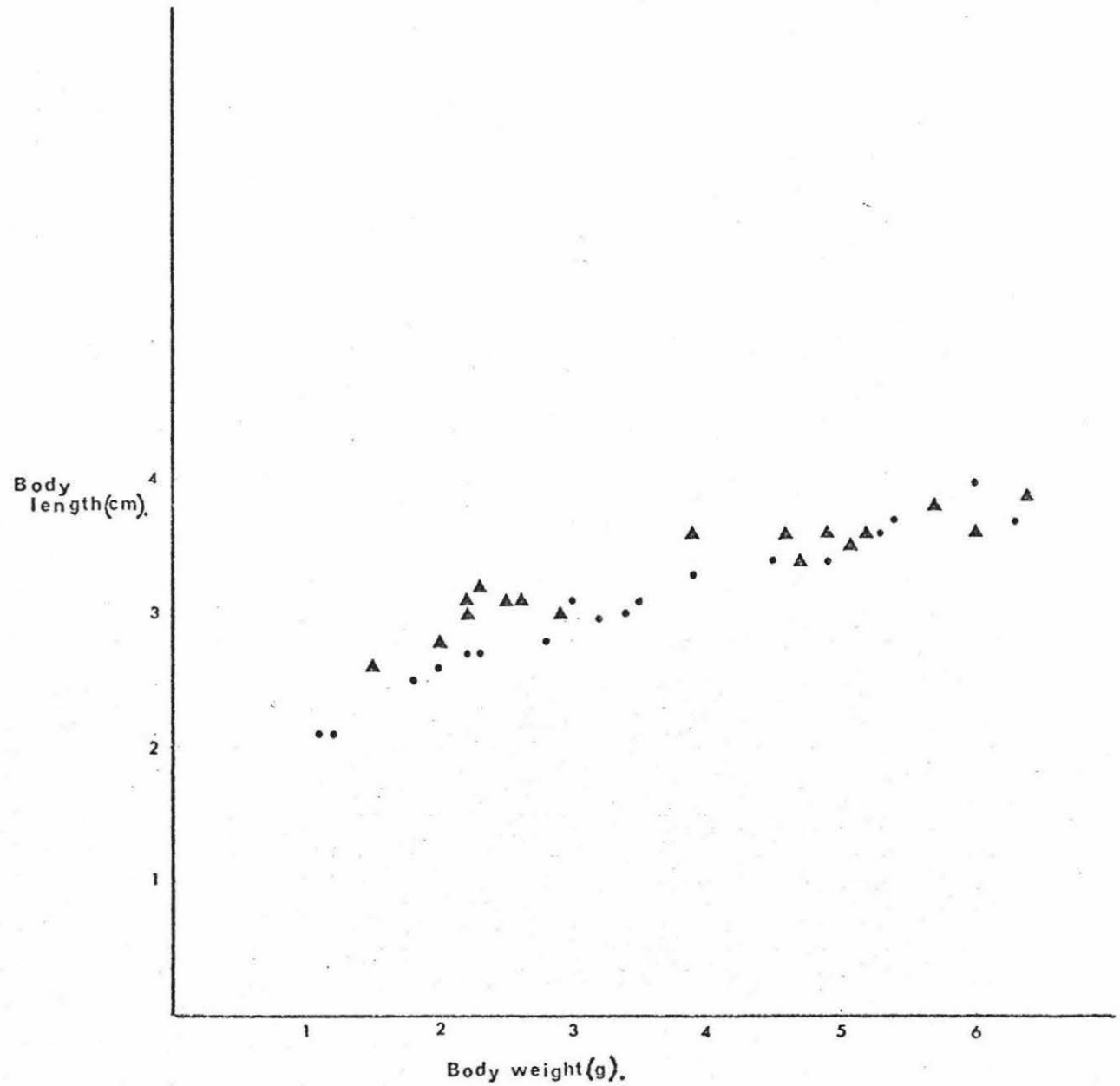
(i.e. not exposed to higher levels of NaCl). The absence of these lipid-transport correlations and a relative increase in sphingomyelin was found to be associated with supression in active Na^+ transport and Cl^- conductance in high salinity conditioning and Watlington *et al.* thought that this could be related to the NaCl transport adaptive process.

Appendix 1.

Relationship between body weight (Standard weight) and body length for two species of frogs.

Li. ewingi ▲

Le. hochstetteri •



APPENDIX 2

Dehydration data. For two species of Frogs,

Li. ewingi

Le. hochstetteri

Expt. No.	Conditions	Unmatched Data Species	wt(g)	H_2O loss ±S.E.	Matched Data Species	wt(g)	H_2O loss ±S.E.
1	11-13°c, 71-77% R.H.	*Li. ew.*	5.45	6.13			
		Le. hochs.	2.19	12.75 ±0.35			
2	11-13°c, 78-84% R.H.	*Li. ew.*	4.88	5.3 ±0.17			
		Le. hochs.	1.39	15.53 ±5.0			
3	10-11.6°c, 83-87% R.H.	*Li. ew.*	5.13	4.55 ±0.35			
		Le. hochs.	1.78	9.87 ±0.95			
4	6-9°c, 85-87% R.H.	*Li. ew.*	2.95	6.48 ±0.5	*Li. ew.*	2.95	6.48 ±0.5
		Le. hochs.	3.58	7.24 ±1.2	*Le. hochs.*	4.27	6.05 ±0.37
5	9-10°c, 80-85% R.H.	*Li. ew.*	3.67	10.59 ±1.2	*Li. ew.*	4.45	9.32 ±2.4
		Le. hochs.	4.29	10.38 ±0.95	*Le. hochs.*	4.31	10.90 ±1.1
6	8.5-10°c, 85-87% R.H.	*Li. ew.*	3.89	6.19 ±1.5	*Li. ew.*	4.33	5.53 ±0.75
		Le. hochs.	4.99	5.54 ±0.3	*Le. hochs.*	4.52	5.57 ±0.4
7	11.5-13°c, 83-85% R.H.	*Li. ew.*	3.74	8.34 ±1.2	*Li. ew.*	2.36	10.28 ±0.4
		Le. hochs.	1.72	11.88 ±1.9	*Le. hochs.*	2.04	10.32 ±0.35

APPENDIX 3

Frog Skin Data.

(a) Numbers of poison (P) and mucous (M) glands per mm^2 in various skin regions (sub totals) of skin

Skin Region	Ventral pectoral		Ventral pelvic		Dorsal pectoral		Dorsal pelvic		Dorsal thigh		Ventral thigh	
Species:	*Li. aurea*											
Frog No.	P.	M.	P.	M.	P.	M.	P.	M.	P.	M.	P.	M.
1	6	69	2	83	13	77	13	69	19	42	0	62
2	18	53	7	52	15	44	19	18	18	13	6	56
3	2	68	9	51	13	43	18	80	16	42	3	90
4	19	89	5	55	24	69	28	77	26	67	8	57
5	26	43	12	28	25	37	27	32	15	47	10	34
Mean	14	64	7	54	18	54	21	55	19	42	5	60
	Li. ewingi											
1	9	34	4	45	32	36	21	54	3	55	7	79
2	2	61			22	64	18	50	8	71	5	67
3	10	49	3	57	25	28	33	16	18	68	15	32
4	1	89	12	73	12	73	15	78	21	68	0	77
Mean	6	58	6	58	23	50	22	50	13	66	7	49
	Le. hochstetteri											
1	10	19	11	16	14	9	11	8	22	16	14	26
2	7	22	8	17	9	16	11	6	24	23	12	44
3	14	23	14	15	16	17	16	11	26	11	16	27
4	10	21	9	18	14	21	15	14	26	24	13	20
5	6	23	7	21	14	28	14	13	22	17	10	36
Mean	9	22	10	17	13	18	13	10	24	18	13	33

(b) Range of skin thickness (mm) in skin regions above.

Li. aurea	.14→.26	.12→.19	.16→.27	.19→.24	.13→.17	.11→.25
Li. ewingi	.06→.16	.06→.33	.11→.15	.08→.15	.06→.10	.05→.20
Le. hochstetteri	.13→.17	.11→.16	.10→.20	.08→.20	.15→.22	.10→.13

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