The Embryology of Lungfish

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Introduction

Interest in the development and phylogeny of lungfish has increased in recent years, partly out of a desire to establish evolutionary links with the land animals, and partly out of genuine interest in these unusual fish. In addition, difficulties faced by the lungfish in the altered environments of the Burnett, Brisbane and Mary Rivers, which are likely to lead to the extinction of wild lungfish in their natural habitats, make it important to record as much information as possible about wild lungfish and their development.

The first description of the external features of development in the Australian lungfish (Semon, 1893) used material from the Burnett River. A later complete description of development in the Australian lungfish was based on material from Enogerra Reservoir (Kemp 1982), one of several introduced localities for lungfish, where adults have now become extinct. Later work has been based on material from the Brisbane River, and there are slight but possibly significant differences between eggs from the Brisbane River and eggs from Enoggera Reservoir, whose source population was the Mary River (O'Connor, 1896). New techniques, such as scanning and transmission electron microscopy and histology based on methacrylate embedded material rather than wax sections, provide additional information for descriptions of lungfish development.

Lungfish development has often been compared with that of amphibia, especially with the development of urodeles, and such comparisons are still being made, for the obvious reasons that the two groups do develop in a very similar way. It is however quite possible that the similarities are related more to the fact that both groups, like many lower vertebrates, produce telolecithal eggs, and their development is similarly constrained by this circumstance, and not because there is a close phylogenetic relationship between amphibia and lungfish. Lungfish development is also similar to the development of other basal fish species, such as Polypterus (Bartsch, Gamballa and Piotrowski, 1997) or Amia (Kerr, 1900), and there may be nothing remarkable in many of the similarities with amphibians. As with other research into vertebrate phylogeny, it is important to make many comparisons, rather than base conclusions on a few limited studies.

The embryology of the African and South American lungfish is not completely understood, These species lay eggs in protected nests or breeding burrows, vigorously defended by the male parent (Kerr, 1950, Johnels and Svensson, 1955), and only a few collections of eggs and young hatchlings have been made (Budgett, 1901, Kerr, 1900, Johnels and Svensson, 1955). Accordingly, most of this chapter is based on eggs, embryos and hatchlings of N. forsteri. The material was collected from several lungfish spawning sites in south east Queensland. Three localities in Enoggera Reservoir were used between 1969 and 1973, while suitable water plants were available in this area for

the attachment of eggs. Several sites on the Brisbane River were also used to obtain material. The observations described in this chapter were carried out on living specimens as they develop in the laboratory, and on light, scanning and transmission electron microscopy of fixed material. All of the techniques are standard and have been published elsewhere (Kemp, 1981, 1999, 2003). Stages of development follow Kemp (1982, 1999). Rearing methods are based on the work of Illidge (1893) and Bancroft (1913), modified by my own experience (Kemp, 1981, 1999).

The egg membranes and the hatching process

The egg of L. paradoxa is enclosed in a thin translucent capsule. This has a soft inner and tough outer layer. The jelly layer that covers the capsule is vestigial, and usually absent (Kerr, 1900). Four proteinaceous membranes, about one centimetre in total diameter, cover the egg of an Australian lungfish. The two innermost membranes are thin and closely applied to each other, together enclosing a spherical space, around 4 mm in diameter, around the egg. They are separated from the surface of the egg sufficiently to allow it to rotate within the membranes. The heavier vegetal pole of the egg rests on the smooth surface of the innermost membrane, but does not adhere to it. Attached to the outer surface of the inner membranes is a dense jelly coat about 2-3 mm thick. This may become colonised by filamentous algae, which penetrate as far as the inner membranes, but no further. Applied to the thick jelly layer is a thin layer of diffuse albumen, responsible for the adhesive properties of the egg when it is first laid. Once this is covered in debris the albumen is no longer sticky, unless the external surface is abraded, exposing a fresh area of the outer coat.

Changes occur in the membranes as the embryo of N. forsteri grows. Eggs and young embryos can rotate within the membranes, under the influence of gravity. There is little increase initially in the diameter of the available space, and in the size of the embryo, despite changes in shape induced by cleavage, blastula formation, gastrulation and development of the neurula. Scanning electron micrographs reveal that the inner membranes of an egg of N. forsteri are folded before cleavage begins. Macroscopic cracks appear in the inner membrane when the fish reaches stage 35, and the embryo is moving more freely (Whiting et al., 1992; Kemp, 1994). As the young fish becomes more active, the inner membranes break into fragments, and fall to the bottom of the sphere defined by the remaining albumen coat. Fragments are small in egg membranes from Enoggera Reservoir, and large in those from the Brisbane River. This is not related to the pH of the water, or to water composition.

During the process of membrane breakdown, the intravitelline space expands further as enzymic digestion of the albumen coat is initiated. The albumen stays intact for about another week. It is continually digested away from the inside, eventually becoming so thin and eroded that the young fish can slip in and out at will (Bancroft, 1913; Kemp, 1987), until it grows too large or moves away too far to reach the membranes easily when startled. Hatching in N. forsteri occurs at around stages 42-43, and the young fish immediately seek shelter among the water plants or rootlets to which the egg was attached. However, embryos of stage 37, or even younger, may hatch, and survive, or the event may be delayed until stage 45.

The process of hatching in L. paradoxa is less elaborate than it is in N. forsteri. A single split appears in the capsule surrounding the egg, and the young fish escapes tail first. The capsule does not expand prior to hatching, and space is constrained (Kerr, 1900), although the young fish is able to move a little and the capsule becomes quite soft. On occasion, the outer membrane ruptures incompletely, and the fish may be trapped for a few days, or retained within the soft inner membrane.

Young lepidosirenids hatch within the protected environment of the nesting burrow, and the event occurs at a much earlier stage of development than in N. forsteri. Young L. paradoxa hatch at stage 27 and Protopterus species hatch at stage 28 (Kerr, 1909). These are equivalent to N. forsteri stages 33 and 34 respectively. The hatchlings are still small, and undeveloped, although they are able to move and are able to cling to the side of the nesting burrow with their cement organ. The young Protopterus is only eight days old (Budgett, 1901).

The fertilised egg

Eggs of N. forsteri are telolecithal, consisting of numerous spherical yolk globules enclosed in a thin plasma membrane or cortex that contains minute melanin granules of egg pigment over the animal pole. The yolk globules are green or brown in colour. Little of the cytoplasm is free of yolk, and most of this surrounds the nucleus deep within the egg. The histology of the fertilised egg is remarkable for the enormous quantity of yolk globules, and the apparent paucity of the cytoplasm that holds them in place. At early stages of development it is hard to distinguish any cytoplasm or cell organelles among the yolk globules that fill the egg. The nucleus is surrounded by a small quantity of cytoplasm, and the cortex encloses the mass of yolk globules. Fine strands of cytoplasm, almost impossible to demonstrate amongst the yolk, link the cortex and the perinuclear cytoplasm.

In N. forsteri, although the fluid filled space enclosed by the inner capsular membranes is spherical, the fertilised egg, 3-3.5 mm in diameter, is not. It is hemispherical, with a flat animal pole and a rounded vegetal pole. The animal pole, with smaller yolk globules, is generally uppermost, and the heavier vegetal pole, with much larger yolk globules, is pressed against the inner membranes. If a living egg is removed from the egg capsule, and placed on a flat surface, it is unable to maintain the hemispherical shape, but spreads over the surface like an unbaked biscuit. In this situation, the contents of the egg are held in by the elastic cortex or plasma membrane.

The cortex, or outermost cytoplasm of the animal pole, contains small granules of dark pigment, so most of the flat surface of the egg is dark in colour. In some eggs, an unpigmented spot, paler than the ground colour of the yolk globules, is present, sometimes central in position, sometimes displaced. An irregular area of intense dark pigment may also be present on the animal pole. Both occur in only a proportion of the eggs collected. Variability in position and occurrence of light and dark spots on the surface of the lungfish egg does not affect subsequent development, such as the position of the cleavage furrows, and this suggests that they have no special function in development of the embryo. The rounded vegetal pole is unpigmented.

Yolk globules in the Australian lungfish are perfectly spherical, and range in size from 2 microns in the animal pole to 20 microns in the vegetal pole. Egg pigment granules are much smaller, 0.1 microns in size. The most common arrangement is an area of pigment in the centre of the flat animal pole of the egg, with small yolk globules below, grading slowly to large globules in the vegetal pole, with no pigment. The largest globules actually lie above the vegetal pole, 20 microns below the surface. In fact globules grade in size around the egg, and show a similar gradation from the outside of the egg to the inside. The distribution and organisation of egg pigment granules and yolk within the egg determines (or reflects) the pattern that cleavage of the fertilised egg will follow.

Eggs of L. paradoxa are spherical, and, at 7 mm in diameter, the largest of the eggs of the living species of lungfish. Kerr (1900, 1901) describes them as holoblastic, salmon pink in colour, with a germinal cap containing small yolk globules that appear white. Granules of dark pigment are absent (Kerr, 1900). Eggs of species of Protopterus are slightly larger than those of N. forsteri, between 3.5 and 4 mm in diameter. The vegetal pole of Protopterus eggs is pale green in colour, with a translucent pink animal pole. Yolk globules are stained by bile pigments in this species (Conant 1977), and the colour of N. forsteri eggs is likely to be derived from the same source. Yolk globules in L. paradoxa are mostly spherical, and in Protopterus species may be elongate or lenticular (Kerr, 1901). In eggs of L. paradoxa, they measure up to 200 microns in diameter (Kerr, 1901).

Cleavage (stages 2-11)

In eggs of the Australian lungfish, the first cleavage is meridional, beginning on the flat animal pole and passing through the egg to the vegetal pole, travelling more slowly as it reaches the more heavily yolked regions. The egg is usually divided into two equal, or nearly equal, halves. The white spot is often not divided in early cleavage stages, and the pigment granules may not be shared equally to the two daughter cells. The second cleavage, which may begin in the animal pole before the first has divided the vegetal pole, is also meridional and at right angles to the first. Each of the neat quarters that form as a result of these two cleavages has some pigment, and part of the pale spot, if originally present, depending on its position in the uncleaved egg. The concentrated darkly pigmented area present in some of the fertilised eggs is no longer apparent. Furrows deep within the egg are often not complete, and this may be apparent on the surface as well. Scanning electron micrographs show that parallel surface wrinkles form in the plasma membrane prior to or during cleavage.

The third cleavage, stage 4, is also meridional, dividing each cell from the animal pole to the vegetal pole, usually radially but occasionally at right angles to the original furrows. Pigment retains its original position on the egg surface, though now divided among a number of cells, often unevenly. The pale spot can still be distinguished. Cleavage furrows are more complete in the animal pole than they are in the vegetal pole. The fourth cleavage, stage 5, is the first latitudinal one, and begins within the confines of the animal pole, the former flat surface. It results in eight small micromeres, uppermost in the intra-vitelline space and with most of the pigment granules and small yolk globules, and eight large macromeres, with little pigment and many large yolk globules. The egg is more rounded on top, and the segmentation cavity or blastocoel has appeared within the

egg. This is placed eccentrically, because of the enormous size of the macromeres in relation to that of the micromeres. Cell size is variable, especially in the vegetal pole.

The fifth cleavage, at stage 6, occurs rapidly in the animal pole and is vertical, dividing the micromeres into numerous small, pigmented cells of different sizes. Asynchronous cell divisions increase in frequency, although division is still more rapid in the animal pole than it is in the vegetal pole. Cleavage in the more heavily yolked regions is slower and may be vertical or latitudinal, producing cells of variable size and shape. The segmentation cavity expands in size as cleavage proceeds.

Although some digestion of yolk globules has taken place, and the scanty cytoplasm of the fertilised egg has increased and been distributed to many more cells, histology of cleavage stages has changed little from that of the fertilised egg. Pigment is still mainly confined to the cortical cytoplasm of the former animal pole, although some has been dragged into the egg with cleavage furrows. Equally, the distribution of yolk globules is essentially the same as it is in the fertilised egg, with the smallest at the animal pole and around the margins of the egg, and the largest in the centre and in the former vegetal pole, with some smaller globules along the cortex of the cleavage furrows. The main change in sections is the presence of the segmentation cavity, or blastocoel. In deeper parts of the egg, the cleavage furrows are often not complete. When cleavage products in fixed eggs are separated, roughened areas on the cell surface are present, indicating the cell membrane is either incomplete or poorly formed. Most of the cytoplasm is still concentrated below the plasmalemma and around the nucleus, and cell organelles are still scanty.

Variations of the normal cleavage pattern are common, and will only impede future development if they are extreme. Small anomalies, such as eggs that form three cells in first cleavage, followed by six in the next cleavage, gradually disappear. Other unusual patterns, such as eggs with gross disparity in the sizes of the first cleavage products, or a first cleavage that is latitudinal, may not survive. The smaller cell divides more rapidly than the larger one, and abnormalities increase as development continues. The pattern described by Semon (1893) as a separate stage, with two large cells separated by two smaller cells in the centre of the animal pole, divided across the centre of the egg, is actually anomalous.

Later stages of cleavage, from stage 7 to stage 9, gradually produce an egg with a sizeable segmentation cavity, and small rounded cells projecting into the space within the membranes. The roof of the segmentation cavity is one cell thick, and most of the egg pigment is concentrated in the plasmalemma of these cells. The floor of the segmentation cavity contains numerous layers of cells, most of these being larger than the cells of the animal pole and all heavy with yolk globules. Some project into the segmentation cavity, and some are free. The external layer of cells of the former vegetal pole consists of polygonal cells of highly variable size in contact with the membranes.

By stage 10, the segmentation cavity has enlarged, and minor anomalies present in earlier stages have been smoothed over. Although so much cell division has taken place, the distribution of pigment granules and of yolk globules of various sizes has not changed from the arrangement found in the fertilised egg, apart from close to the cell

membranes, and there is still surprisingly little cytoplasm present in the separate cells. If a white spot was present in the fertilised egg, it is still distinguishable on the surface of the former animal pole. Up to stage 10, the egg is able to rotate within the membranes. Short cytoplasmic processes extend from the plasmalemma to link adjacent cells.

During the formation of an egg of stage 11, cell division continues as before, and the segmentation cavity (blastocoel) enlarges considerably, so that the egg fills the space within the membranes and is no longer able to rotate. Fluid is transferred into the blastocoel from the surrounding space, until the egg resembles a little balloon. The roof of the blastocoel is distended, and consists of a single layer of columnar cells. A mass of large heavily yolked cells forms the floor, and there is no sharp transition in cell size in the former animal pole and the former vegetal pole. In eggs prepared for scanning electron microscopy, the roof of the blastocoel collapses.

Cleavage in eggs of L. paradoxa proceeds in a manner similar to that of the eggs of other lower vertebrates, such as Petromyzon, Amia, Acipenser and Triton, and is affected by the large size of the egg and the quantity of yolk globules in the vegetal pole (Kerr, 1900, 1901). The first cleavage furrow divides the mass of pale yolk globules in the animal pole in two parts, and the second forms at right angles to the first while the first furrow passes towards the equator of the egg. Both furrows extend to the vegetal pole, while additional vertical furrows form in the animal pole. After a number of vertical cleavages, which become increasingly irregular, latitudinal furrows form. Cleavage is more rapid in the animal pole than in the vegetal pole. Eventually, the egg is converted into a spherical blastula with a large segmentation cavity roofed by a single layer of cells. During the process of segmentation, the cells on the surface of the egg are close to the egg capsule. Cells within the cavity are rounded and in close contact with each other. Small cells of the animal pole have spread downwards to the equator of the egg, where the layer of cells is double. There is a sharp transition between the cells of the animal and vegetal poles. The late cleavage stage is spherical, like the egg, and the blastula, prior to the invagination of endoderm and mesoderm, is similar to that of N. forsteri.

The earliest stage of development of Protopterus obtained by Budgett (1901) is an egg in mid cleavage. This process in the eggs of Protopterus resembles that of eggs of L. paradoxa, but the outer cells of the animal pole are rounded and not in contact with the egg capsule, as in N. forsteri (Budgett 1901). Cells of the animal pole are smaller than cells of the vegetal pole, and, as in eggs of L. paradoxa, there is a marked transition between them.

Gastrulation and formation of the neural folds (stages 12-24)

Gastrulation in N. forsteri follows a slightly different path to the same process in L. paradoxa and in species of Protopterus. The shapes of the blastopores at successive stages differ. However, the blastopore in all three genera is surrounded by the posterior neural folds, and ultimately becomes the cloaca (Budgett, 1901; Kerr, 1900, 1901, 1909; Kemp, 1982; Semon, 1893).

In N. forsteri, early stages of invagination of endodermal and mesodermal cells take several days to complete, and mesodermal cells continue to enter the embryo via the blastopore during formation of the neural plate. Gastrulation shows as much variability as cleavage, and in the same way, minor anomalies disappear and the more extreme anomalies of the process do not survive. Embryos with less severe anomalies eventually complete the process successfully.

The process is initiated at stage 12 in N. forsteri when a slightly curved slit appears just below the junction between the cells of the expanded blastocoel and the more heavily yolked cells of the former vegetal pole. Exact position is variable. Cells forming the roof of the segmentation cavity begin to invaginate, and draw in the more heavily yolked cells below the slit. The opening develops into a crescentic blastoporal lip as cells move into the interior of the embryo, around stage 13. Heavily yolked cells continue to divide and are pulled in by the cells of the dorsal lip. By stage 14, cells on the lateral margin of the crescent are also actively moving, and more and more cells with egg pigment are entering the embryo from the roof of the blastocoel. Lateral lips draw together, forming a rhomboidal shape at stage 15. The ventral lip is slow to appear, and does not form until cells of the former vegetal pole have all disappeared into the interior of the embryo, leaving only pigmented cells on the outside. This produces a slit shaped blastopore by stage 18, as the neural folds are beginning to form. Posteriorly, the neural folds enclose the blastopore. The blastocoel contracts during the process of cell invagination, and the embryo is once more free to move within the intra-membranous space.

The blastopore, early in formation of the neural folds, varies in shape from a dorsoventrally oriented slit to a small oval cloaca. By means of active migration, cells, mesodermal and endodermal in type, continue to enter the embryo through the blastopore. By this time, formation of the neural folds and of masses of anterior mesoderm is already well advanced (Greil, 1908, 1913). As the embryo continues to develop, it becomes increasingly difficult to place the different developmental stages into categories. Early stages of neurula formation overlap with the later stages of gastrulation, and, by the time the nerve cord has closed posteriorly, parts of the brain and sense organs are already forming anteriorly.

Gastrulation in embryos of L. paradoxa and Protopterus species differs in several ways from the condition found in N. forsteri. The process begins in Protopterus with an irregular slit between the small cells derived from the animal pole of the egg, and the larger yolk laden cells of the vegetal pole. The slit is initially straight as cells begin to move inwards, and later curves. Lateral and ventral lips become delineated around the large cells (Budgett, 1901, Pasteels, 1962). This contracts to a small opening lying sideways across the egg, and ultimately enclosed by the neural folds as they form around it. Embryos of L. paradoxa pass through a similar process, starting with a long line of invaginating cells, extending around one third of the circumference of the embryo, and finishing with a small upwardly curved blastopore, which lies within the neural folds (Kerr, 1900, 1909).

The blastopore of dipnoan embryos does not develop into a large round yolk plug (Kerr 1900), characteristic of amphibian embryos and apparently found in embryos of

Polypterus senegalus (Bartsch et al., 1997). A large yolk plug may be found in N. forsteri embryos from the Brisbane River, but this is not normal (Kemp, 1994).

Medullary folds

Development of the medullary folds in all three genera of living lungfish follows a similar pattern, with minor variations that are related to the way the embryo fits within the egg capsule. In N. forsteri of stage 16, the embryo is free in the capsule, and the area where the neural folds will form is flattened, although the blastopore has yet to close. By stage 17, the presumptive neural plate is delineated by a double streak of darkly pigmented cells, most obvious anteriorly, which extend back to the blastopore. The anterior neural folds begin to rise by stage 18, and by stage 19 the folds are elevated, with numerous cells containing egg pigment inside the folds. The folds are wide in the region of the developing brain, and there is a distinct transverse crest. Folds are low posteriorly, and closer together. A shallow median furrow runs between the neural folds. This is the stage when the embryo may have an ectodermal median suture, a deep bilateral curving invagination in the position of the furrow. This was described by Semon (1901) in eggs from the Burnett River. It occurs rarely in specimens from Enoggera Reservoir, and never in material from the Brisbane River.

In embryos of stage 20, the transverse crest is deeper, and three bilateral swellings, corresponding to the three primary vesicles of the brain, are already present, although the neural folds are widely separated. These are more obvious in fixed material, but still present in living eggs. Vesicles of the developing brain become more distinct as the folds grow and draw together at stage 22, and as the transverse crest sinks inwards at stage 23. By this stage further division of the fore and hindbrain is evident. The folds are in actual contact at stage 24, and epithelium is beginning to cover the folds anteriorly. The process continues in an anteroposterior direction, with the folds still open posteriorly at stage 25.

Development of the neural folds begins in L. paradoxa with a slight depression extending forwards from the blastopore, as in N. forsteri. Medullary folds form from this depression, close together medially and open anteriorly and posteriorly. The folds are less obvious in L. paradoxa than they are in N. forsteri, because there is less space within the capsule. They meet in the midline. However, according to Kerr (1901) the neural rudiment is solid, because it is pushed into the surface of the embryo by the closeness of the egg membranes. There is little obvious differentiation of the brain into regions, so marked in N. forsteri. As the neural folds develop, the blastopore contracts to a small triangular opening and gradually forms the cloaca. There is no neurenteric canal in this species. The embryo at this stage is curled around the mass of endoderm cells, and closely confined in the egg capsule.

Formation of the neural folds in Protopterus is similar to the process that unfolds in L. paradoxa. Development is most obvious anteriorly and extends back to enclose the blastopore. The folds are less elevated than they are in N. forsteri.

Cleared specimens and sections of developing embryos of the Australian lungfish reveal that the neural tube is hollow from the time of formation (Kemp, 1982), unlike the

equivalent structure in the South American lungfish, which develops as a solid cord that sinks in to the embryo and only later becomes hollow (Kerr, 1900, 1903). The cavity in developing embryos of the Australian species may be very narrow, but the arrangement of the cells, and the concentration of the granules of egg pigment in these cells, indicates that it is a hollow nerve cord from the earliest stages of development. In places, especially along the spinal cord, no cavity is apparent in sections, but the cells radiate from a small central point.

The mesoblast

In embryos of N. forsteri, below and lateral to the developing anterior neural folds, a mass of mesodermal cells, are visible in section (Greil 1908, Kemp 2000), and in scans. The two blocks of cells meet in the mid line below the forebrain and appear to flow forwards. These are equivalent to the “neural crest migration” described in older embryos (Falck, Joss and Olsson, 2000). The mesodermal cells appear early, and increase in number and density as development proceeds. No migration from the neural folds has appeared at this stage (Kemp, 2000). The sequence of development of the mesoblast in N. forsteri is illustrated in detail by Greil (1908, 1913).

Scans of stage 25 and 26 show that the neural folds are intact, without migrating cells, and many cells are present around them. The first indication of migration of cells from the neural tissues occurs in embryos of stage 27, and is confined to cells moving towards target organs, initially the otic capsule and nerves in the vicinity, possibly cranial nerves V or VII. These cells spill from the dorsal surface of the nerve cord, and are smaller than the cells of the mesoblast on either side of the nerve cord. As development proceeds, further cells migrate to form additional cranial nerves.

Head and tail buds

As the head and tail buds form and grow, differences between lepidosirenid embryos and those of N. forsteri become increasingly apparent. Neural tissues are completely covered by epithelium in embryos of N. forsteri by stage 25, except at the posterior extremity. The primordium of the auditory placode is present at stage 26. The opticoele is visible as a swelling covered by skin, and the head is elevated above the endodermal mass, as the branchial eminence develops. Egg pigment has concentrated in the cells that will form neural tissues, and neuromeres are evident in the anterior neural cord. As the posterior medullary folds fuse and the epithelial cover forms over them, the tail fold appears. Growth of the head continues over the next six stages, with the oral and pharyngeal regions increasing in size and becoming more defined. At stage 31, the head is still curled around and touches the globular endodermal mass, and the embryo is unable to extend within the membranes. By stage 32, vigorous movements of the embryo, accompanied by dissolution of the membranes from within, have enlarged the space within the capsule and the head is free to move away from the endoderm. Few external marks of the active developments in the brain and sense organs are visible externally, although the thin roof of the hindbrain is evident, the eye protrudes more and gill slits are visible in cleared embryos. The head continues to extend forwards away from the globular endodermal mass. At these stages, the tail bud is small.

Formation of the head and tail buds in L. paradoxa may appear similar to N. forsteri until stage 25, with the embryo curled around the mass of endodermal cells. However, when the head begins to grow away from the endoderm, the tail extends more rapidly, leaving a round mass of yolk laden cells below the head, and the tail free. In N. forsteri, the head grows forwards from the endodermal cells of the developing intestine. Formation of the head fold and mesoblast alters the contours of the head region and the brain is less obvious. A tail fold also appears as the medullary folds fuse. Both folds are obvious by stage 23, equivalent to stage 28 in N. forsteri. The embryo is able to move two days before it hatches, and the tail straightens after the embryo leaves the confined space within the egg capsule. Embryos of Protopterus undergo a similar sequence of development.

Development of the eye

An opticoele is apparent as an outgrowth of the forebrain in N. forsteri as early as stage 23, and distinct by stage 25. Tissues of the developing eye are close to the epidermis, and a lens is induced and incorporated in the usual way, as the optic stalk lengthens. The eye is obvious externally by stage 30, and pigment appears in the retina at stage 35, just before pigment cells appear in the rest of the body.

The opticoele is apparently a little late to appear in L. paradoxa, becoming evident at stage 21 (Kerr, 1901), equivalent to stage 26 in N. forsteri. The opticoele in L. paradoxa and Protopterus is solid when it first appears. As in N. forsteri, pigment appears first in the eye.

Epiphysis

The epiphysis of N. forsteri embryos is apparent at stage 32 as a small bulbous outgrowth of the diencephalon. This structure grows into a small sphere connected to the midbrain by a short stalk. It consists of large cup shaped neurons with axons radiating from the adjacent midbrain, enclosed by glial cells and epithelium. The epiphysis is attached to the rostral surface of the brain, just below the skin. At this stage, the forebrain and developing olfactory organs are in a ventral position.

Development of the auditory system

In N. forsteri, the auditory placode is visible at stage 25 as a shallow depression, lined with cells containing egg pigment, on each side of the head. The depression develops into a recessed pit during stage 26 and sinks inwards at stage 27, eventually closing over. A nerve forms dorsally and makes contact with the auditory primordium by stage 26, before the placode closes. The ductus endolymphaticus is present by stage 34. The membranous labyrinth, enclosed in cartilage on either side of the hindbrain, is present in young fish of stage 50.

The auditory placode of L. paradoxa, according to Kerr (1900), develops by secondary excavation of a primordium that is originally solid, a few days after hatching. This primordium forms from the inner layer of the ectoderm, and a cavity develops within the

rudiment, to create a thin walled sac, which is still connected to the ectoderm. The process is similar in Protopterus (Budgett 1901).

Myotomes

Mesoderm enters the embryo of N. forsteri early in gastrula formation, and myotomes are already present in the mid-dorsal region while the neural folds are developing. The mesoderm is undivided in embryos of stage 18. Up to five myotomes, mid dorsal in position, are present in embryos of stage 21. A variable number of myotomes, up to eleven, are present by the time the neural folds have closed. Anteriorly the mesodermal block has no myotomes, and sweeps around, below the developing brain, to form the musculature of the upper jaw and mandible, as well as skeletal structures in the head, and other connective tissues. Myotomes are added anteriorly and posteriorly until stage 24 then only posteriorly as the body and tail extend. From around stage 33, a V-shape is present in myotomes in the middle of the trunk. Before this stage, the myotomes are oblong (Greil, 1913). Differentiation of the cells within the myotomes to form contractile tissues occurs at stages 28-29.

Mesodermal cells contain yolk globules of sizes intermediate between those of the ectoderm and nervous system, and those of the endoderm. Metabolism of the yolk in these cells proceeds more slowly than it does in the ectoderm, but more rapidly than it does in the endoderm. As in cells of the nervous system, granules of egg pigment are found in mesoderm cells, especially in the myotomes. The concentration of egg pigment in cells of the myotomes delineates them almost as clearly as it does the neuromeres.

Development of myotomes occurs as the medullary folds close in L. paradoxa (Kerr, 1900). The number of myotomes increases rapidly as the tail fold extends, and is variable among embryos of similar stages of development. The V-shape is present from stage 30.

The Fate of Egg Pigment

Eggs of N. forsteri have minute granules of melanin, or egg pigment, mostly in the cortex of the animal pole. The granules are likely to be protective in an egg that is laid within open water, and often in full sunlight (Kemp 1984, 1987). It is absent in eggs of L. paradoxa, which are laid deep in a breeding burrow in dark peaty water (Kerr, 1900). It is present but not copious in eggs of Protopterus species (Budgett, 1901). These are also laid in a nest, often in shallow water and open to the surface.

Egg pigment, originally concentrated in the animal pole, is included mainly in ectodermal cells as they divide, and concentrated within the neural folds as they develop. Many are found in developing myotomes as well However, egg pigment can be transferred from cell to cell within the embryo, and may turn up in cells of the archenteron or notochord, that are endodermal in origin, as well as in cells derived from ectoderm or mesoderm. It is not a reliable indicator of the origin of the cell.

Pronephros

Early development of the pronephros in N. forsteri follows an amphibian pattern (Goodrich, 1930; Kerr, 1919) and is comparable to development in other living lungfish. The pronephros helps to provide landmarks of development in early stages of head development, as it begins to form at stage 27, when it is visible as a prominent bulge below the skin posterior and ventral to the auditory placode. In cleared embryos and in scanning electron micrographs, it is associated with post-otic somites 4, 5, and 6. In stage 32 it has two tubules and a short duct, which extends towards the tail by stage 33. By stage 35, the entire organ has moved posteriorly in relation to the somites. After this stage it is less easy to distinguish in whole specimens.

In L. paradoxa, the pronephros appears as a slight elevation on each side of the medullary folds, which are in contact but not yet fused, and a little in front of the middle of the embryo. As the somites are developing, the duct of the pronephros grows posteriorly. A little later, at stage 22, it develops a comma shape at the anterior end, reflecting the development of pronephric chambers.

Cement organ

A major difference between young of the lepidosirenid lungfish species and N. forsteri is the presence of a cement gland, or cement organ, also found in the embryos of Anura. This forms in the deeper layer of the ectoderm prior to hatching, at stage 23, in both L. paradoxa (Kerr, 1900) and in Protopterus (Budgett, 1901). This becomes crescentic in shape and the outer layer of the ectoderm breaks down just before hatching. Originally spread across the ventral surface of the head, it becomes more compact after the embryo hatches and reaches a maximum size about a month after hatching. The cement organ is glandular, and is used by the young fish to cling onto the sides of the nesting burrow. As the young fish grows and becomes more active, the cement organ undergoes atrophy. It is completely absent in N. forsteri hatchlings.

The archenteron and the origin of the notochord

The archenteron develops in relation to the inward movement of cells at the lips of the blastopore, a process that obliterates the blastocoel or segmentation cavity. Cells that will form the notochord are initially a part of the roof of the archenteron. The notochord forms when a block of medial cells delaminates from the archenteron, in L. paradoxa (Kerr 1903), and the roof of the developing intestine closes over the space. The process is underway in embryos of stage 20, and the nerve cord over the partially formed notochord has already begun to differentiate anteriorly. More posterior regions of the archenteron still include the notochord as part of the roof.

The notochord in embryos of all three living genera of lungfish develops by delamination anteriorly from the roof of the archenteron while the process of invagination of endodermal and mesodermal cells is still in progress through the posterior blastopore Kemp, 2000; Kerr, 1901). Notochordal tissues differentiate rapidly as mesoderm condenses on either side, and the basic structure of a sheath surrounding vacuolated cells is present while neural tissues are forming.

Development of the oral cavity.

As with other fish and possibly some amphibians, much of the oral epithelium is actually derived from endoderm (Kemp, 2003). Forward growth of the presumptive foregut brings endodermal cells into contact with the ectoderm of the stomodaeal plate by stage 25, although the oral cavity is not yet apparent externally. Development of the oral cavity is apparent as early as stage 29, with the pale endoderm, laden with yolk, visible through the thin stomodaeal plate, a flattened area of epithelium below the developing brain. From stage 30, it is associated with the appearance of the olfactory placodes. Externally, by stage 35-36, the oral region is defined by the developing upper lip, with the olfactory placodes well anterior to it, and indistinct lateral and mandibular regions surrounding the pale endodermal cells. A lower lip is evident at stage 38, and this gradually grows forwards to meet the upper lips at stage 44. The oral cavity grows deeper slowly as the lips form more fully, gradually incorporating the developing olfactory organ, first as part of the upper lip, then within the oral cavity.

As the neural tube is forming and closing, the foregut endoderm makes contact with the primordium of the ectodermal stomodaeal plate. This has two layers of cells when it first develops, and the endoderm cells are highly oriented, as long columnar cells extending from the stomodaeal plate to the cavity of the foregut. As the embryo grows, the outer layer of stomodaeal cells disappears, and the oral cavity grows deeper while the foregut extends towards it. Foregut cells consist of a single layer of elongated cells with the basal surface against the inner layer of epithelial cells. Eventually the second layer of stomodaeal cells disappears, foregut and oral cavities grow deeper, and the two cavities join at around stage 42-44. The oral epithelium associated with the developing dentition is initially heavily yolked and the epithelium of the lips is not. A gradual process of digestion of the yolk in the cells of the oral cavity removes all traces of the cell origin of the epithelium in the oral cavity, and structures characteristic of the epithelial cells surrounding the mouth, such as a brush border and desmosomes with tonofilaments in the cell membrane, appear in the endodermal cells as the dentition begins to develop (Kemp, 2003).

The oral cavity of L. paradoxa develops in a similar manner to that of N. forsteri, and involves ectodermalisation of endodermal cells (Kerr, 1903). However, development of the characteristic dentition of L. paradoxa is precocious in the extreme, and fusion of separate cusps to form the tooth plate occurs before the oral cavity has formed. As in N. forsteri, dental tissue is mesoblastic in origin (Kerr, 1903).

Development of the olfactory system

The external openings of the olfactory organ of N. forsteri appear before the oral cavity actually forms. At stage 30, a distinct, shallow depression in the epithelium on the lateroventral surface of the head is present, anterior to the stomodaeal plate and to one side of the midline, angled to the long axis of the head and body. The primordia of two openings are visible at each end of the oval pit, and ciliated cells are present in the depression. The anterior opening is more distinctive than the posterior. The opening visible externally is associated with bulbous swellings of the inner epithelium on each side of the forebrain. By stage 33, the posterior opening in the primordium has elongated into a slit running towards the developing oral cavity. By stage 36, the

olfactory opening appears as a single, deep pit, drawn further inwards towards the oral cavity. Eventually, the primordium, now apparently single, lies within the upper lip. As the oral cavity and the dentition begin to form, outgrowths of the epithelium divide the olfactory primordium into two parts superficially, possibly reflecting the original division in the organ. The two openings then grow away from each other. In older fish, the anterior opening of the olfactory organ is situated on the innermost margin of the lip, rostral to the vomerine teeth and visible when the mouth is closed. The posterior opening lies on the labial margin of the pterygopalatine tooth plate, at the level of the second ridge. Cilia are present within and around the openings, particularly anteriorly, and within the olfactory organ are large stereocilia. At no time is the opening of the olfactory organ associated with the development of the bones of the jaw. It develops in soft tissues anterior and lateral to the developing skeleton. Outgrowths of the trabeculae cranii enclose and protect the associated sensory tissues that have developed from the forebrain.

In L. paradoxa, the olfactory primordium arises as a solid mass arising from of the deep ectoderm, and a cavity appears within the primordium as it develops (Kerr, 1903). The opening of the olfactory organ forms in a manner akin to that of N. forsteri. As the internal cavity develops in the olfactory tissue, a dimple is present on the external surface. This develops into a slit shaped opening on the ventral surface of the olfactory rudiment, oriented outwards and backwards, and within the developing lips. The opening is dilated anteriorly and posteriorly, and fusion of the narrow passageway between these dilations produces an anterior and a posterior opening (Kerr, 1909). The olfactory system of Protopterus species develops in a similar manner (Kerr, 1909).

Development of the gills and the operculum.

As the head of an embryo of N. forsteri begins to develop, a prominent pharyngeal eminence develops behind the foregut, and two developing gill clefts are present, visible in cleared embryos of stage 30 (Kemp, 1982). This number has increased to 4 by stage 33, and 5 by stage 34. However, the clefts do not yet open to the exterior. At stage 36, the operculum appears as a fold of skin between the first, or spiracular, gill cleft, and the second, the first true respiratory gill slit. The operculum has covered the second and third gill slits at stage 38, and all four of the posterior clefts by stage 41. The gill openings are never visible externally at any time. The operculum forms as an epithelial fold growing back over the gill primordia before they open.

Analysis of the ciliary current through the oral cavity at stage 42 indicates that the gill slits are open at this stage. Opercular folds meet in the ventral midline at stage 44, a connection that persists into late hatchling life.

Young hatchlings of stages 43 and 44 have short, blunt gill filaments below the operculum. These consist of a loop of capillaries covered by densely ciliated epithelium. The slender filaments are few in number and circular in cross section at these early stages. Gill filaments are never external in this species, as the gill slits are covered before the filaments develop. Nor are the original gill filaments lost to allow the gill filaments of the older fish to develop. As with other structures in the lungfish hatchling,

the structures present in the adult are derived from a process of gradual change in the young fish.

In subsequent stages, the gill filaments increase in number and become compressed, with many more cilia evident on the epithelium. They are a little longer than they were at stage 44, but remain simple in structure and do not protrude beyond the operculum. By stage 50, the filaments are densely packed in double rows on the anterior and ventral surfaces of each gill bar, and the cilia are showing signs of degeneration. The filaments do not regress, and buds of the adult gill lamellae appear, first on the more anterior filaments and progressively involving the posterior filaments. This change begins at about stage 51, and takes place over several months. By the time the fish is a year old, the gill lamellae are densely packed, and almost free of cilia, as they are in adult fish.

A current of water in drawn through the mouth as soon as the foregut joins the oral cavity, and this passes over the gills when the gill slits become patent. This is a respiratory current, and not a feeding current. Lungfish do not feed by filtering microscopic plants and animals from the water at any stage of the life cycle.

The possession of external gills is a notable and obvious difference between the young of N. forsteri and those of lepidosirenid lungfish. They appear in embryos of L. paradoxa at stage 25, as dorsolateral outgrowths of the four branchial elevations, visible in earlier stages on either side of the anterior medullary folds (Kerr, 1900). They increase in size, and develop feathery lamellae soon after hatching. The four gills, one on each side, appear to arise from a common base. The four large blood vessels that supply each gill are visible in living hatchlings, parallel to each other under the skin of the neck, and the opercular fold appears below and anterior to the base of the first gill. A favoured position of the hatchling at this stage of post hatching life is to lie in a sloping position among the detritus on the floor of the nest with the head sticking out and the external gills, which are freely moveable, spread wide. External gills reach their greatest size at stage 35, at which time the hatchling is more active and capable of breathing air. They atrophy from this point, and disappear at around 40 days post-hatching.

In Protopterus the external gills begin to develop just before hatching, and grow rapidly. As in L. paradoxa, the external gills can be raised and lowered depending on the position of the hatchling and the activity. They reach their maximum size earlier than those of L. paradoxa, and disappear more slowly (Budgett, 1901).

In L. paradoxa, the opercular fold grows posteriorly from stage 32, to form the opercular cavity. This covers the gill slits, which are ventral in position and still closed. At about stage 35, while the external gills are maximally developed, the gill slits within the opercular cavity become patent. Gills in adult L. paradoxa are almost vestigial, with no respiratory lamellae, and in the adult, only four open gill clefts (Kerr, 1909). The clefts and supporting tissues reduced in comparison with N. forsteri. The operculum of Protopterus develops to cover the gill slits before the slits are open, and the internal gills are functional (Budgett, 1901).

The fate of endodermal cells

The presumptive foregut makes contact with cells of the stomodaeal plate by stage 25, and behind the foregut cavity the archenteron becomes wider before narrowing as it arches over the endodermal mass below. At stage 27, the posterior extremity of the archenteron grows wider, and is linked to the cavity of the nerve cord by the neurenteric canal, which does not close until stage 34. As the trunk extends the archenteron grows longer, curving over the globular endodermal mass. After stage 32, as the body extends, the endoderm becomes pear shaped, with the narrowest diameter at the anterior end. The cells surrounding the archenteron remain heavily yolked until well after hatching, and are blocked into platelets delineated by blood vessels, which show under the thin epidermis. These were described by Semon (1893) as developing scales, but the scales do not in fact appear until much later. The platelets are particularly obvious in embryos of stages 37-38, before pigment in the dermis has developed. Yolk in the endodermal cells is metabolised continuously, and significantly reduced in quantity by the time the young fish hatches. It is still obvious by the greenish colour at stage 47, when the blood vessels delineate the forming spiral valves of the intestine. By stage 49, when the spiral valve is well formed, the yolk globules are no longer grossly obvious, although a few can still be found within the intestinal cells. The large anterior pocket of the intestine, the first part of the gut to receive food, has acidic contents, and the more posterior spirals gradually become more alkaline.

Most of the yolky endodermal cells in Protopterus and in L. paradoxa are concentrated below the head region, behind the cement organ, and blood vessels passing over the endodermal cells much more obvious (Budgett, 1900; Kerr, 1909). As the tail extends, and the yolk is used up, a spiral valve with a large anterior chamber develops (Budgett, 1901, Kerr, 1900).

Development of the heart and cardiovascular system

Heart development has no unusual characteristics in N. forsteri embryos. A cardiac field is present in embryos of stage 28, and contractility of the developing heart is visible by stage 30. Red blood cells within the developing circulation are present by stage 36. Movement of red blood cells is visible below the skin, especially over the endoderm, at stage 37, and cells entering major veins on either side of the developing intestine and under the skin below the sensory line system of the trunk, can be followed by stage 40. By stage 43, the platelet like arrangement of the endodermal vessels loses definition, and the major blood vessels of the gut, visible below the thin and partially pigmented skin, follow the spiral valves of the intestines. A sparse and irregular pattern of capillaries is present below the skin of the tail from stage 40 onwards.

Differences in the heart and circulation of lepidosirenid lungfish are largely related to the presence of external gills and the blood vessels associated with them, and in the greater development of blood vessels over the developing intestines. This suggests a possible respiratory function of these vessels, in contrast to the condition in N. forsteri.

Skin

Differentiation of the skin in N. forsteri embryos proceeds rapidly after the neural folds are covered. Two layers of cells have developed by stage 26, and the outer layer is

squamous. Cells at this stage are similar in external appearance. By stage 31, a few cells are developing cilia, and the surrounding cells are beginning to develop a brush border. By stage 44, when ciliation of the skin is at its height, two other cell types have made their appearance. One type has a brush border, and the other has a rough outer membrane.

The hatchling skin has many unusual properties. Although the epidermis is only two cell layers thick, it is tough and impervious, protecting the fish from chemical and mechanical insult in its environment. It contains numerous superficial cells that produce slime, some cells with a sensory function and, in young fish prior to and for a month or so after hatching, ciliated cells. Differentiation into several cell types early in development is evident by the different surface morphologies of the cells. Most of the external cells have a brush border, shown by striations in the light microscope. In addition, the skin of a young N. forsteri is a transporting epithelium (Kemp 2003).

Like the skins of young stages of some invertebrates, the skins of young N. forsteri are capable of conduction of an electrical impulse (Bone et al., 1989). This is most easily demonstrated in lungfish embryos prior to hatching, at stage 35-36, and before any nerves have developed under or near the skin The value of this ability to the young fish is uncertain, as it is unlikely to have anything to do with the mediation of an escape response before the fish is free living.

The epidermis of the developing lungfish skin lacks pigment in early stages, as does newly formed skin, for example around the margin of the tail as the fish grows. Melanophores appear first in the retina, at stage 37, and slightly later in the skin over the dorsal surface of the body. These extend gradually towards the ventral surface, covering the entire body by stage 52. There is no hard line between the pigments and unpigmented cells, as is found in lepidosirenid hatchlings (Budgett, 1901, Kerr, 1900). Cells containing pigment are originally confined to the dermis, between the epidermis and a thick layer of collagen. As development proceeds, some melanocytes invade the layers of epidermal cells. Chromatophores containing a pink pigment, perhaps ferritin, appear at stage 40. Young hatchlings are capable of responding to light conditions by contracting and expanding the melanophores, but lose this ability once the pigment is dense. The mottled pigmentation of juvenile lungfish is a developmental stage, and is strongly affected by the conditions in which the juvenile lives.

In L. paradoxa, the two layered ectoderm of the young fish develops into the multiple layers of the adult, and glandular cells begin to develop. Pigment in the skin appears first, in both L. paradoxa and Protopterus, in the retina, and then on the dorsal surface of the body, beginning in Protopterus, on the anterodorsal surface of the head (Budgett 1901). Pigmentation of the skin in L. paradoxa develops around 12 to 15 days after hatching. The newly hatched fish is a pale yellowish salmon colour (Kerr, 1900). By stage 30, at 10 days after hatching, the embryo is white except for the yolk laden endoderm, which is yellow and shows through the skin. Pigment is appearing in the eye at this stage, equivalent to an N. forsteri embryo of stage 38. Within 24 days after hatching, the young fish has fully pigmented eyes and a dense scatter of chromatophores over the dorsal surface of the body. These spread ventrally, with a sharp division between pigmented and unpigmented skin, during subsequent stages,

and cover the whole of the body by stage 38, by which time the dorsal surface is uniformly dark. A similar sequence of events occurs in Protopterus (Budgett, 1901). Hatchlings of lepidosirenids are capable of responding to changes in light conditions, as in N. forsteri.

Ciliated epithelium

Embryos of N. forsteri, and young hatchlings, have cilia in the external layer of epithelial cells. Endodermal cells, which form the lining of the intestines and the coverings of the gill filaments, are also capable of developing cilia, in the oral cavity, on the gills and in the intestines. A ciliated epithelium is not unusual in the young of invertebrates and lower vertebrates. Cilia in the epithelium of L. paradoxa have not been demonstrated. Budgett (1901) states that movement, unrelated to movement of the embryo, is visible within the egg cases of Protopterus, and it is probable that the skin of these embryos is ciliated.

Timing of appearance of cilia in young of N. forsteri, and their eventual disappearance, is variable. Cilia appear in ectodermal cells in embryos around 10 days after fertilisation, and at least 10 days before hatching ls likely to occur. Cilia at these early stages are short, sparse and of simple construction. They are scattered randomly over the cell that has produced them, and the surface of the cell from which the cilia arise is not differentiated. Ciliated cells are most numerous on the dorsal surface of the embryo, especially in the groove between the endoderm and the body axis. There are few cilia on the head, and none on the skin covering presumptive oral region or the endoderm. Few cells in these early stages have any cilia, but the arrangement of the cells that have developed cilia is already apparent, oblique to the long axis of the body. Cells surrounding the cloaca are heavily ciliated.

As the embryo begins to move within the egg capsule, ciliated cells proliferate, especially dorsally and extending over the head. A few are found ventrally, in the oral region or the olfactory placodes. Within each ciliated cell, the cilia grow in a dense mat, aligned obliquely across the long axis of the cell and surrounded by cell membrane that is free of cilia. The cell membrane differs in appearance to the membranes of surrounding cells, having numerous short projections instead of irregular hexagonal pits. Cilia are now longer and denser.

Embryos of stages 34-36 have ciliated cells all over the body, and these are most dense on the head and on dorsolateral parts of the trunk, except on the tail bud. As before, the cilia are oriented obliquely across the cell, and similarly arranged across the body axis. They are arranged in specific tracts across the external epithelium of the body, and are now present in the presumptive oral cavity and around the anus.

The density and complexity of structure continues to develop between stages 38-40, as the time of hatching approaches, especially in the head region. Cilia are particularly copious in the developing oral cavity, around the olfactory openings, on the lip primordia and on the ventral surface of the head. Ciliated cells are now highly organised and the cilia are long and dense, and there is no difference in structure among the ciliated cells of different parts of the body.

By the time the embryo is ready to hatch, cilia have attained a peak of structural complexity and density in the skin. Cells bearing cilia are separated by only two or three cells without cilia, and are even more abundant in and around the skin covering the blind oral cavity. At stage 44, when density and activity reaches a peak, ciliated cells are arranged in a pattern oblique to the long axis of the fish, and each ciliated cell carries a dense row of cilia, diagonally across the length of the cell. The cell itself has a reticulated pattern on the surface, and each cilium arises from one of the spaces in the reticulum.

After stage 45, the number of ciliated cells on the external epithelium declines, and the highly structured arrangement of the cilia on the cell surface loses definition. By stage 48, as the activity of the young fish begins to increase, the ciliated cells that remain are smaller and fewer in number than the cells without cilia, and the number of cilia that they carry is reduced. Ciliated cells are lost first from the skin overlying the lateral line of the trunk, and then from ventral regions of the body.

Before the mouth becomes patent, the oral epithelium is densely ciliated. Once the oral cavity joins the foregut, the cilia disappear, except around the openings of the olfactory organs. By stage 45, only a few are found on the lips, and none on the oral epithelium surrounding the tooth plates and covering the jaws. Scattered ciliated cells are present on the skin of the tongue, until stage 48, and many remain around and within the openings of the olfactory organ, particularly the anterior opening. The lining of the opercular cavity is still heavily ciliated, as are the gill filaments. Cilia within the oral cavity are not associated with the gathering of food material, and there is no sign of an apparatus capable of gathering food particles, similar to the structures present in filter feeding animals such as amphioxus.

Cilia in the epidermis of lungfish have two central filaments surrounded by nine paired filaments beneath an outer limiting membrane. Cilia arise from a basal body embedded in the cell below the outer membrane. The basal body, or axoneme, includes horizontal striations, reflecting areas of high and low electron density. It is enclosed in a membrane. No nerve connection has yet been demonstrated.

Prior to stage 31, ciliated cells do not create movement of material over the cell surface. From stage 31-33, in eggs that are still within the egg capsule, or have been removed from it artificially, ciliary action creates movements that run along the groove between the endoderm and the skin covering the somites, and the current runs along the embryo from head to tail. At stage 34, particles may be flicked out of the main stream, in a dorsal or a ventral direction. Some are removed from the surface in this way, and others collect around the vent or along the margin of the developing dorsal fin. Heavier particles do not move, especially on the belly. As the embryonic axis lengthens and the endodermal mass loses its rotund appearance, the major movements created by the ciliary currents are diagonal, moving particles off the dorsal and ventral surfaces of the embryo. As the mouth forms, particles stream into the cavity and out, where they join the flow of material over and around the dorsal and ventral surfaces of the head and into the stream of material flowing diagonally over the surface of the body. These movements take place in the absence of muscular movements in the animal, or under the skin. This pattern of

movement continues after the oral and foregut cavities join, and, as before, heavier particles collect on the margins of the fins and around the cloaca. Matter does not accumulate on anterior regions of the body.

As the oral cavity grows deeper, particles are swept in and out. After the gill slits and the mouth become patent, virtually all of the particles that enter the mouth are swept out under the operculum. Fragments that impinge on the head away from the mouth are removed over the skin of the head, not directed into the mouth. No attempt is made to gather material from around the head, even if it is microscopic plant matter that could serve as food. Occasionally, inorganic matter may enter the intestine, perhaps because it is too heavy to be taken up and removed by the ciliary current over the gills. After the cilia in the oral cavity have disappeared, particles still enter the mouth and leave via the operculum, driven by the ciliary currents in the opercular cavity, or perhaps by muscular movements of the jaws.

Sensory lines, ampullary organs and pit lines.

Lungfish have a complex system of sensory lines, pit lines and ampullary sense organs, particularly on the head, and concentrated around the jaws and on the snout. These are vital to a fish with poor vision that lives in tannin stained and frequently cloudy water, but are not well formed when the young fish hatches.

The lateral line of the trunk in N. forsteri hatchlings appears at stage 40, as a raised papilla below the epidermis, behind the primordium of the operculum. The papilla extends down the trunk between the epaxial and hypaxial musculature, leaving a shallow, unpigmented groove in its wake. By stage 43 it has reached the tip of the tail. There is a slight flexure at the tail tip, which disappears as the fish grows. Neuromasts appear, anteriorly at first, and subsequently along the length of the trunk, evenly spaced and in the base of the groove, which closes over. They are not arranged in line with segments of the body. A line of smaller ampullary pits form above and below the lateral line. Externally these have a single opening, and are not surrounded by modified skin cells. Initially, the skin over the lateral line is ciliated, but these disappear after the young fish hatches, leaving a streak of skin without cilia over the sensory region.

Initially, the sensory (lateral) lines of the head consist of shallow grooves lined by epithelium devoid of melanophores. Neuromasts develop in the base of the grooves. Numerous single ampullary pits and pit lines appear later, after the sensory lines form, particularly under the skin of the snout and the mandible. After stage 47, the neuromasts sink beneath the surface of the skin, and are linked by a canal. Ampullary pit organs and pit lines may be close to the sensory line canals, but are not derived from them. Additional sense organs develop in the skin of the head, especially in the lips. These consist of tubules packed with cells.

Lateral line organs appear early in the development of L. paradoxa, and are already present at stage 27 when the young fish hatches (Kerr, 1909). The neuromasts are arranged segmentally, and retain their original position on the surface of the skin. In Protopterus, they sink inwards and run below the surface (Kerr, 1909).

Sense organs within the oral cavity

In addition to the olfactory organs, taste buds are scattered over the oral epithelium, including the epithelium around the tooth plates and the skin covering the hyoid apparatus. These consist of a raised papilla of cells with a hair cell at the apex. They begin to develop soon after the oral cavity joins the foregut. The paired vigidial organs (Bartsch, 1992) are present in the upper lip. Each consists of an elevated papilla of epithelial cells, with a cluster of sensory cells at the tip. They resemble the gustatory organs of the oral cavity, but are larger.

Development of the paired fins

Primordia of the pectoral fins appear in hatchlings of N. forsteri at stage 42, as minute unpigmented protrusions below the skin of the trunk, one on each side immediately behind the free edge of the opercular folds and slightly below the developing sensory line. Pigment cells soon appear in the skin covering the fin primordia, and they grow rapidly, soon attaining the typical rhomboidal shape of the N. forsteri pectoral fin. By stage 45, the operculum has grown backwards to cover the anterior third of the fin, and this appearance does not change even in adults. Skeletal structures within the fin are visible by stage 47, and fins are moveable by stage 48. Pelvic fins develop in a similar manner but appear later, between stages 48-50.

The pectoral girdle, made up of cartilage and bone, begins to form as a cartilaginous primordium at stage 45, and is still cartilaginous when the fins become functional. This encircles the body just behind the head. Formation of the bone is, as in other parts of the skeleton that develop from a cartilaginous template, perichondral. Layers of bone are laid down around the cartilaginous template, but no replacement of cartilage by bone occurs. The pelvic girdle is entirely cartilaginous at all stages of the life cycle, and is embedded in the tissues of the ventral body wall anterior to the fins.

Pectoral and pelvic fins are important in feeding behaviour of N. forsteri hatchlings from an early stage, and are used to brace the body against the substrate while capturing prey animals. During slow locomotion, they are used in an alternate movement, as in sharks, in Polypterus and in higher vertebrates.

Pectoral and pelvic fins develop synchronously in lepidosirenid lungfish, and are always long and slender with minimal development of fin rays in Protopterus and none in L. paradoxa. The pectoral fin develops just below the external gills. Young of L. paradoxa use the hind fins to clamber among submerged water plants (Kerr, 1900).

Development of the medial fins

Medial fins are important at all stages of the life cycle, and provide the major propulsive force for the lungfish particularly during rapid swimming. At all times during development, the tail is diphycercal in all three genera of living lungfishes (Kerr, 1900). Bemis (1984) regards the tail as protocercal, and a sign of paedomorphosis in dipnoans.

In embryos of N. forsteri, a tail bud forms at stage 28 as the myotomes and body axis start to develop, but does not grow significantly until the body extends and yolk in the endodermal cells is reduced, around stages 33-35. By stage 36 a median dorsal fin, one third of the length of the trunk, has appeared. A short median ventral fin appears at stage 37, and the dorsal fin extends forwards. At its maximum extension, at stage 38, this fin reaches the back of the head, although it regresses in later stages and extends only as far as the middle of the trunk by stage 53. A ventral fin on the belly anterior to the cloaca is present from stage 38, and this reaches its greatest development by stage 49, after which time it regresses, disappearing completely by stage 53. Skeletal elements are present in the dorsal and ventral fins posterior to the anus, but these never develop in the pre-anal ventral fin.

In lepidosirenid lungfish, dorsal and ventral medial fins appear soon after the young fish hatches, and grow rapidly (Kerr, 1900, Budgett, 1901). Fin rays soon appear, particularly in the dorsal fin (Budgett, 1901). A pre-anal ventral fin fold is present in both L. paradoxa and Protopterus, but this is small and soon disappears. It does not affect the posture of the hatchling, which can remain upright because the cement gland holds it in position.

Movements

The egg of N. forsteri is able to move within the intravitelline space but only under the influence of gravity, because the large heavy yolk globules of the vegetal pole move to the lowest possible position within the membranes. The lightly yolked animal pole is uppermost. In L. paradoxa, and the egg rests with the animal pole underneath the large yolk globules of the vegetal pole (Kerr, 1900). Eggs and embryos of L. paradoxa cannot move within the membranes. Mobility of the N. forsteri egg is lost just before gastrulation when the segmentation cavity swells to fill the entire egg capsule, and regained as formation of the medullary folds begins. Protopterus eggs and embryos have limited space (Budgett, 1901).

As the N. forsteri embryo develops, muscular movements of the head region, consisting of a rapid side to side flick, develop. These appear stage 32 (Whiting, Bannister and Bone, 1992). Embryos are able to move the tail similarly at stage 35-36. A similar movement is possible in L. paradoxa embryos prior to hatching, but the embryo is so confined within the egg capsule that little movement is possible.

Young embryos of N. forsteri, removed from the egg membranes, and young hatchlings, are capable of a rapid escape movement, using the side to side flick, without attaining a normal dorsoventral posture. This is an undirected response to extreme stimulation of the fish, and is apparently exhausting. Rapid swimming, with the body held in a dorsoventral posture, and using the tail fin as well as the body musculature, develops when the fish is old enough to assume a normal dorsoventral position, at around stage 50. Slow movements using the tail and the paired fins appear gradually as the fins develop, and are established by stage 52. At stage 50, the paired fins are large enough to be used to brace the body against the substrate during feeding.

Posture and Activity

When the young N. forsteri hatches, it is almost completely inactive, and spends most of the time under laboratory conditions lying on one side, at least during the day when it can be continuously observed (Bancroft, 1913). It does not move unless disturbed by strong stimulation. If the hatchling has access to a clump of filamentous algae or other water plants, or to the egg case, it can rest in a more upright position, but it is not able to maintain this posture without support This is partly because of the pre-anal ventral fin on the abdominal mid-line, and partly because the paired fins are short. A dorsoventral posture develops gradually as the pectoral fins begin to grow, and the pre-anal ventral fin regresses from stage 49 onwards. By stage 50, the young fish no longer needs to make use of external supports to maintain its posture.

Young hatchlings of L. paradoxa are also inactive for some time after hatching, and rest almost continuously in the protected environment of the nest (Kerr, 1900). In this they are aided by a large cement organ, not present in N. forsteri hatchings.

Respiration

Although the air breathing habit is never frequent in N. forsteri, unless they are stressed in some way (Kemp, 1987), the capacity to inhale air through the mouth develops in hatchlings. Young lungfish start to breathe air at around stage 52, under laboratory conditions, and may do so in the wild at an equally early stage of development. The Australian lungfish is not normally exposed to hypoxic or anoxic conditions. Hatchling lungfish are said to rest with the mouths out of water, (Bancroft 1918), but this activity is not related to respiration, nor is it necessary for the young fish to do so. It is done before the lung is functional, and older lungfish like to rest in banks of weed in a similar fashion. Breathing air does not involve the olfactory organs, which are purely sensory (Atz, 1952).

Air breathing develops in young L. paradoxa while the external gills are still large and functional (Kerr, 1900). In Protopterus, air breathing develops about one month after they leave the nest, when the external gills are lost (Budgett, 1901).

Colours

Hatchling lungfish vary in colour, depending on their environment, and the colours are cryptic. When newly hatched, N. forsteri are green ventrally, because of the yolk in the endoderm, with brown and pink dorsally, good for hiding among vegetation or submerged tree roots. Young of L. paradoxa vary in shade from dark grey to black, with yellow spots. Protopterus hatchlings are dark brown, with a "conspicuous yellow band between the eyes" (Budgett, 1901). All three species are capable of contracting the melanophores in the skin, under various conditions, to render themselves less conspicuous. Chromatophores with pink or yellow pigment do not contract (Kerr, 1900).

Initiation of scales

Among living vertebrates, initial appearance of lungfish scales on the trunk most closely resembles that of teleost fishes (van Oosten, 1957; Sire, Allizard, Babiar, Bourguignon and Quilhac, 1997), in that the scales of most teleost species first appear near or below

the sensory lateral line of the trunk. As in many teleosts, there is an additional site of formation of scales on the ventral surface of the head, remote from any sensory line.

The first row of scales, on the trunk of the young fish, forms below the sensory line of the trunk late in stage 52 or early in stage 53, beginning one third of the way from the pelvic fin to the pectoral fin. The scales develop anteriorly and posteriorly from the point of initiation, and rows are added alternately below the line, and above the line, until they reach the dorsal or ventral mid-line, or fin membranes, at about stage 56. Scales develop on the ventral surface of the head, from about stage 56, from a separate centre, and appear on the lateral and posterior dorsal surface of the head by extension of scales on the trunk and under the lateral line.

The large elasmoid scales of the Australian lungfish, Neoceratodus forsteri, are formed within the dermis by a capsule of unpigmented scleroblasts, growing within a collagenous dermal pocket below a thick glandular epidermis. In early stages, the scleroblast capsule is in contact with the epidermis over the posterior quarter of the scale, but the cells that form the capsule are dermal in origin. As the scale grows, it is enclosed in a collagenous pouch that covers the whole scale, and separates the posterior scale from the epidermis. The collagen pouch and the epidermis remain closely associated.

Feeding

The young of Protopterus species and N. forsteri are carnivorous when they first begin to feed, and will be cannibalistic if given the chance. Hatchling Protopterus start to feed soon after they leave the nest, about 3 weeks after spawning. When Budgett tried to bring a number of young Protopterus home from the Gambia to London, a long journey in those days, he arrived home with a single juvenile, which had consumed all of its companions (Budgett, 1901). Kerr (1900) does not discuss feeding and raising young L. paradoxa.

Many attempts have been made to raise young N. forsteri, and most of these failed because it was not realised that the young are active carnivores when they start to feed (Bancroft, 1913). In many cases, the food offered was not appropriate for young fish (Semon, 1893). If offered suitable live animal food, such as daphnia, brine shrimp or tubificid worms, hatchlings of N. forsteri begin to feed at around stage 46, before the yolk has disappeared from the endoderm, and almost as soon as the jaws are able to move. Fish reach this stage at about four weeks after hatching, under laboratory conditions, and growth and postural changes follow rapidly. Later, at around stage 54, the diet of the young fish changes to include plant material if this is available in their tanks. Older lungfish will choose an omnivorous diet.

Assumption of the adult form and behaviour

Among vertebrates, metamorphosis is variable in occurrence (Fritsch, 1990). It is not surprising that the two major divisions of living lungfish differ in whether they undergo metamorphosis, with the process absent in N. forsteri, and present to a varying degree in lepidosirenid lungfish hatchlings.

The Australian lungfish does not undergo metamorphosis, and all of the changes related to growth, development and the assumption of the adult form and behaviour are gradual, including changes in the gills, dentition, skin and sensory line system of the head. In common with many other fishes and numerous other vertebrates (Fritsch, 1990), a sudden change in structural characteristics, of any tissue or organ, or in any function, does not happen in N. forsteri. After stage 34, the globular shape of the endoderm begins to change as the body extends and the median fins appear and grow. The young lungfish has a thin trunk when viewed dorsoventrally, making the head and pectoral fins appear over large. This outline changes slowly as the fish grows and adult body contours are formed. The marginal dentition of the lower jaw is resorbed between stages 51 and 53, cilia are gradually lost from the gill lamellae over a period of months, beginning at stage 51, ciliated cells in the epithelium decline in number and activity from stage 44, the single openings of the neuromasts in the sensory lines of the head, present in young juveniles, gradually give way to multiple openings in the adult fish.

Kerr (1900) comments that, about six weeks after hatching, the larva undergoes rapid changes, which one might almost designate by the name "metamorphosis". The young fish becomes more active, and pigment in the skin becomes denser and covers the whole body. External gills atrophy quickly and disappear. Around this time the young fish leave the shelter of the nest. Changes in Protopterus are less dramatic (Budgett, 1901). Both species can be considered to undergo metamorphosis in the usual sense.

Discussion

Reproduction and ontogeny in living lungfish shows many adaptive characteristics, and many similarities with each other and with other early vertebrates. There is however, little consistency, and comparisons may be drawn with fishes such as Polypterus, and with different groups of amphibia. In particular, oviposition has a strong relationship to the environment. Protopterus species, and L. paradoxa live in swamps and slow flowing rivers, and lay their eggs in protected burrows or nests. The water in the nest is aerated by the male parent, and the eggs are protected until the hatchlings leave the site. The Australian lungfish lives naturally in flowing rivers, and can breed in large lakes that are not used as reservoirs and have permanent macrophyte beds around the shore. Many details regarding the choice of site by the lungfish are still obscure despite many years of research. Eggs may be shed into the environment close to macrophytes or to submerged tree roots, to which they then become attached, or they may be placed in among the plants. There is no parental care, and more than one pair of fish may use a single site.

Certain aspects of egg membrane structure are clearly adaptive. The inner lining of the egg capsule in all three genera of living lungfish is similar, and consists of a coat of clear protein. This is double in N. forsteri, and may be so in the other two genera. A thick jelly coat, important for the attachment of eggs to the water plants, is always present in N. forsteri, and its presence is variable in eggs of L. paradoxa. Young L. paradoxa hatch when a single spilt forms in the capsule. In N. forsteri, the process is complex, and the inner layer breaks into fragments held in place by the outer jelly. The hatchling does not escape from the jelly for at least another week, and uses this as a shelter for several

weeks after hatching, under laboratory conditions and in the wild (Bancroft, 1913; Kemp, 1987).

Cleavage and formation of the segmentation cavity in lungfish eggs is constrained by the amount of yolk in the egg, as in a number of other early vertebrates such as Amia, Acipenser, Polypterus, Petromyzon, and in amphibia. It is complete, unequal, and slow in the more heavily yolked parts of the egg. The segmentation cavity develops by the coalescence of intercellular spaces. The cellular layer covering the cavity is initially single, and becomes double as gastrulation begins.

Details of the gastrulation process in lungfish are not well understood. Gastrulation proceeds by invagination of large cells in the vegetal pole of the egg. Significant differences between the two lungfish groups are the initial formation of blastopore, and the shape of blastopore ultimately formed. In lepidosirenids, a long line of invaginating cells forms a small crescent shaped blastopore, enclosed within the medullary folds, and in N. forsteri a short crescentic line of invaginatiopn produces rhomboidal blastopore, contracting to a dorsoventral slit, also enclosed within the medullary folds. The slit is continuous with a depression, sometimes an infolding, along the base of the medullary folds. The final shape of the blastopore in lepidosirenids is similar to that of Petromyzon, in N. forsteri closer to amphibia. In all three genera, the blastopore becomes the cloacal opening. There is a neurenteric canal in N. forsteri, but not in lepidosirenids. As the tail bud develops, neural tissues behind the blastopore disappear.

Development of the medullary folds in L. paradoxa, and to a lesser extent in Protopterus, is controlled by the tight egg capsule. In these species, neural tissues form as a solid keel, similar to the process in teleosts. Development of the medullary folds in N. forsteri resembles that of amphibia, and the tissue has space to expand upwards and for a neural tube. As in the other genera of lungfish, development in the medial regions is slightly in advance of the anterior and posterior sections, and the tube forms first in the middle. Primary vesicles of the brain are easily recognisable in N. forsteri, and in all three genera myotomes are forming on each side of the nerve cord at the same time.

Formation of the cranial nerves (neural crest) follows the pattern of teleost embryos and is identical with that of L. paradoxa (Kerr, 1909). Around stage 25, an irregular mass of cells appears in the dorsal midline of the developing nerve cord. There are few cell divisions. The disordered arrangement of these cells is in stark contrast to the highly ordered cells in other parts of the developing brain and spinal cord. The cells, containing few small yolk globules and numerous granules of egg pigment, migrate and form cranial and spinal nerves as the target organs develop. Before this migration takes place, cells, with a pattern of yolk granule content typical of mesoderm, have proliferated in the developing head, specifically in the region of the jaws and gills, to form skeletal, dental and muscular structures. This copious mass of mesoderm cells, heavy with yolk globules, unlike ectoderm and neural tissues of the same stage, flanks the developing neural tissue, but is not of neural origin (Greil, 1913; Kemp 2001). It forms muscular, dental and skeletal structures of the head, and involvement of “neural crest”, if any, comes from the innervation of head structures.

Hatchlings of L. paradoxa and Protopterus develop four sets of external gills, on branchial arches I-IV, high on either side of the developing brain. These are richly supplied with blood vessels to numerous pinnae, and capable of being extended or moved to lie alongside the body. Gill clefts, below an operculum, and within an opercular cavity, form later, and are more ventral in position, as the external gills are beginning to atrophy. Gills in L. paradoxa are reduced. Young of N. forsteri never have external gills, and the gill clefts develop below the operculum, opening into the opercular cavity but never to the exterior. Gills of N. forsteri are heavily ciliated in the young fish, and these are lost as lamellae develop.

Newly hatched lepidosirenid lungfish have a glandular cement organ, which allows the hatchling to attach itself to the walls of the nest or to detritus. Young Anura also have a cement organ. This structure is absent in N. forsteri. The auditory sac is initially solid in L. paradoxa, and a cavity forms during development. In N. forsteri, it forms as a placode in the skin and sinks inwards. The nasal sac in both species forms as a solid block of cells, with a cavity developing later. Formation of the external opening of the olfactory organ follows the same path in all three genera, with a single slit inside the roof of the mouth dividing into two openings, which grow away from each other. Early indications of the olfactory opening in N. forsteri are double, but this disappears as the slit forms and sinks towards the oral cavity.

Despite numerous claims to the contrary, the olfactory organs of the lungfish are not associated at any time with the respiration of air (Atz, 1952). The anterior opening within the upper lip, the posterior opening between the vomerine and pterygopalatine tooth plates, and the sensory structures within the olfactory organ are equivalent to the olfactory organ of other fish, and to the olfactory region of the tetrapod nasal cavity. No part of the lungfish olfactory organ should properly be described as nares or nostrils, and it is purely olfactory in structure and in function (Atz, 1952). When lungfish draw air into the lungs, they do so through the mouth, and the olfactory organ is not involved.

The skin of all three genera develops in the same way, pigment patterns are also similar, and hatchlings of all three genera can contract the melanophores in respose to differing light conditions. Young N. forsteri have many ciliated cells in the skin, before and after hatching, and these may also be present in Protopterus. The function of the cilia is disputed. They may be there to provide movement of water over subcutaneous blood vessels, operating in a countercurrent mechanism for the uptake of oxygen, as in some species of teleost larvae (Liem, 1981). The lack of many blood vessels under the skin of N. forsteri, and the presence of functional external gills in Protopterus, suggests that this is not the case. Another possibility is that the ciliary current gathers food particles (Whiting and Bone, 1980). However, the cilia are present well before the young fish hatches and starts to feed, and there is no apparatus inside the pharynx to collect filtered particles (Kemp, 1996). The direction of flow induced by the cilia suggests that they keep the young fish clean in a dirty environment (Kemp, 1996).

Hatchlings of all three genera form a spiral valve in the intestine in a similar manner, and begin to breathe air several weeks after hatching. They are initially supine and quiet when they hatch, lepidosirenids in a nesting burrow and young N. forsteri in a protected environment among water plants close to shore. Hatchling N. forsteri will also use the

vacated egg capsule as a shelter for several weeks after hatching. Young of all three genera of lungfish become more active and start to feed at similar stages of development, and are always more active at night.

Early development in lungfish of all three genera closely resembles that of other basal vertebrates, including several amphibians, as well as Polypterus, Amia, Acipenser, and Petromyzon. In some ways, there are more differences among the genera of living lungfish than between lungfish and other vertebrates, such as cement glands and external gills in one group, and a cavity in the medullary folds in the other. As such, it is difficult to use morphological characters of developing lungfish to support one or another phylogenetic argument.

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