A LIFE-HISTORY APPROACH TO THE EARLY ONTOGENY
OF <u>OREOCHROMIS MOSSAMBICUS</u> (PETERS)

THESIS
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by
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Erratum Sheet

MSc Thesis: 'A life-history approach to the early ontogeny of
<u>Oreochromis mossambicus</u> (Peters)'   by KK Holden

1.   p. vi, line 10: 'Humphrey' to read 'Humphry'.

2.   p. 1, 2nd para: To avoid confusion, it should be noted that the
     'new trend' in the various academic disciplines lies in their
     adoption of an holistic approach. The term for that approach,
     'holistic', dates, however, back to Smuts (1926). Also,
     'Smutts' to read 'Smuts'.

3.   p. 37, 2nd para, last line: insert 'to be' after 'appeared'.

4.   p. 61, 2nd para, line 7: 'mucous' to read 'mucus'.

5.   p. 70, line 5: delete comma after 'rapid'.

6.   p. 77, Figure 24, last line of legend: 'scl = sclera' to read
     'scl = supracleithrum'.

7.   p. 84, 3rd para, line 5: 'tilapine' to read 'tilapiine'.

8.   p. 85, 2nd para, line 5: 'is' to read 'are'.

9.   p. 89, in section of step $F^1 8$, line 4: 'replacment' to read
     'replacement'.

10. p. 91, 2nd para, line 13: 'criteria' to read 'criterion'.

11. p. 92, line 3: 'embryos' to read 'larvae'.

12. p. 93, line 1: 'ecological' to read 'eco-ethological'.

13. p. 113, 7th reference: 'SMUTTS' to read 'SMUTS'.

ABSTRACT

The adult ecophenotype of an animal is the result of interactive events which occur during its early ontogeny through a series of sequential steps of increasing morphological complexity. At the step boundaries, there are qualitative and quantitative changes in the structures present which will allow the developing animal to successfully cope with new trophic features of its internal and external environment. There is some controversy as to whether the shifts that occur at these step boundaries are gradual or saltatory. Environmental parameters, early life-history plasticity and genetic instructions are the interactive events which produce the ecophenotype. The early ontogeny of Oreochromis mossambicus was followed from the time of egg activation until juvenilization. The eggs, embryos and larvae were artificially incubated at $25\pm0.5°C$. The descriptive text and the figures are composites derived from drawings, photomicrographs and observational notes of live and preserved individuals. It was found that the early ontogeny of O. mossambicus consists of an embryonic period of approximately 11 days and a truncated larval period of about 4-5 days. The embryonic period can be further divided into a cleavage, an embryonic and a free-embryonic phase. Comparisons of the early development of three other mouthbrooding and one substrate-spawning cichlid done by other researchers, revealed that the early ontogeny of O. mossambicus closely resembles that of Sarotherodon melanotheron (also a mouthbrooding species). Embryo length and yolksac area changed gradually in the cleavage and embryonic phases, showed little or no change in the free-embryonic phase, and changed rapidly in the larval period. Heartrate increased gradually in the embryonic phase and peaked just before the shift into the free-embryonic phase. Gradual decrease in heartrate occurred in the free-embryonic phase followed by a second peak at the boundary between the embryonic and larval periods. In the larval period, heartrate levelled off. Although distinctive boundaries were not

clearly evident at all steps, it was concluded that there are three definite ontogenetic steps which occur at the boundaries between the embryonic phase, the free-embryonic phase and the larval period. These thresholds occur at a point where shifts in the trophic features of the external environment are expected. Regression analysis revealed that two regimes exist for changes in embryo length and yolksac area and that a breakpoint occurs just prior to the boundary between the embryonic and larval periods. It appears that ontogeny is saltatory at points where a change in the trophic features of the environment requires a new set of interactions to occur between the environment and the ecomorphological and eco-ethological features of the animal.

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CHAPTER 1:   INTRODUCTION

Aims and objectives

This is not a treatise on the development of any particular tissue, organ or organ-system; neither is it meant to map out minute details about early ontogeny.   It is a broad overview of the interactive events that occur during the embryonic, larval and, in part, the juvenile periods in the life-history of one fish species, Oreochromis mossambicus (Peters) 1852 (Cichlidae, Teleostei).

There is a new trend emerging in the world of science which transcends any particular discipline.   It does not even limit itself to the realm of science, for that matter, and has permeated into the social sciences, the humanities and the arts.   Commonly used terms for this approach are holistic and ecological (Smutts 1926).   An ecological approach incorporates the holistic concept, but the converse does not necessarily apply (Capra 1988). Both approaches incorporate and illustrate the entire picture rather than concentrating on certain isolated elements without considering the interactions between the parts. The holistic viewpoint considers the entity as an integrated whole rather than the mere sum of its parts.   The ecological approach takes this attitude one step further and considers the inter-relationships of the entity with other whole systems or entities.   Some knowledge or awareness of the contributing components is necessary to understand the final product, but the study of minute details of those parts is not necessarily beneficial, as has formerly been thought.   If one looks too closely at the part without relating it to the whole, one runs the risk of losing sight of the importance or impact of the object of initial interest. This attitude has been adopted by many modern conservationists who feel that the strategy of preserving the integrity of an ecosystem has an advantage over one-species preservation programmes. The penultimate example of this holistic approach to conservation is inherent in the aims, objectives and

strategies of the International Union for Conservation of Nature and Natural Resources (I.U.C.N. 1980).

Both of these approaches were adopted in conducting this research. The examination of the minute details of the early ontogeny of O. mossambicus was not the objective. The intent was to follow the development of this species in its entirety, and to explore how the unfolding ontogenetic events related to each other and how their interactions affected the functional aspects of the structures present. Also of importance was the ability of the organism not only to maintain itself at a given level of development, but to move up the hierarchical ladder of morphological, physiological and ethological complexity. An additional objective was to relate how the pattern and rate of development of O. mossambicus reared in the laboratory under simulated natural conditions, reflects the ability of this species to cope with the unique conditions of its expected natural habitat. The relationship between the ontogenetic events and the evolution of the life-history style and the reproductive guild of this species was also examined. To a lesser extent, speculations were made regarding the underlying mechanisms which allow a single cell to develop into a highly organized and complex life form.

Historically, much embryological and larval research has been done using dead and preserved specimens mainly for taxonomic and descriptive purposes. This type of work has centered largely on producing fate maps and explaining developmental changes using sectioned material, thereby producing a disjointed overview. However, the early ontogeny of any organism is a dynamic, ever-changing process. More recent experimental studies have attempted to understand not only the processes themselves, but the causal effects as well (Cohen & Massey 1982). Often this information has not been taken out of the realm of the laboratory and related to the ecology and ethology of the creature in its natural

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environment. However, in fish development some exceptions do exist, such as the work of McElman & Balon (1979, 1980), Paine & Balon (1984a, 1984b) and Cunningham & Balon (1985, 1986). The majority of this type of work has been done on temperate species and not on tropical or sub-tropical fishes. Only a few comprehensive studies of the development of African fishes have been conducted such as Fishelson's (1966) and Balon's (1977) works on cichlids and Haigh's (1989) work on cyprinodonts.

Saltatory ontogeny, homeorhetic states and early-life history patterns

The theory of saltatory ontogeny (Balon 1984a) postulates that development occurs in a step-wise fashion, whereby populations of cells, tissues and structures grow at a rate such that their functional completion is synchronized at a particular point. A rapid switch in form and function then occurs that allows the organism to interact with its internal and external environments in a different way, e.g. as in cellular differentiation and first exogenous feeding, respectively. This sequence of steps (homeorhetic states) of relative stability and thresholds of instability, continue throughout the life of the organism but is of greater frequency and importance in early ontogeny. Greenwood (1989) disagrees and suggests that ontogeny is a process of stepped gradualism and that the apparent saltations are accelerated intervals along a continuum of progressive morphological complexity. Whether ontogeny is saltatory or not can be tested by undertaking detailed embryological studies. Such studies could also shed some light on the underlying processes, their causal effects and the means by which such information is transferred from generation to generation.

Major components of the environment which can dramatically affect the ability of an aquatic organism to survive are predation

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pressure, and oxygen and food availability. These factors are likely to be of even greater importance during the very early life-history stages of an animal. Kryzhanovsky and Balon (see Balon 1981) proposed that predation pressure and oxygen availability are important during the embryonic and larval periods of fish and influences how a species copes with them. Relationships between morphological and behavioural characteristics of fish embryos and larvae and the demands of their natural habitat, appear to exist.

Animals exhibit both intra- and inter-specific differences in life-history styles and phenotypes according to the environment they inhabit. Some environments undergo cyclic or predictable perturbations. These expected changes constitute a stable, predictable environment which remains near equilibrium or returns to it after disturbances (Bruton 1989). Residing within this more equable and competitive ecosystem are more specialized, equilibrium species which are density-dependent. In marginal, unpredictably perturbed ecosystems there are opportunistic species which undergo rapid population growth during short favorable conditions and are density-dependent. The ability of an organism to utilize and survive environmental flux is largely dependent on the life-history style that it has evolved. Several alternative life-history patterns have been described, such as r/K-selection (MacArthur & Wilson 1967), altricial/precocial (Balon 1981) and maintenance/dispersal phenotypes (Geist 1989). Certain suites of characters define which life-history style an organism has adopted. Any particular life-history pattern is not necessarily useful for all types of animals or circumstances. Because of its emphasis on early ontogenetic and reproductive characteristics, the altricial/precocial life-history pattern was utilized for this study. Nice (1962) and Ricklefs (1979) used the terms altricial/precocial to explain alternative forms of neonate birds and Balon (1979, 1981) adapted these concepts to fish.

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Characteristics of the early life-history of species at the precocial end of the altricial-precocial continuum are small numbers of large, highly nutritious eggs, well-developed, large young at first exogenous feeding, direct development and extensive parental care. In contrast, the altricial suites of characters are numerous small eggs, smaller size and less developed embryos at first exogenous feeding, indirect development and no parental care.

Phenotypic and life-history style plasticity are also major contributing factors to the success of an animal in its ecosystem, regardless of the predictability or unpredictability of its environment. The expression of alternative morphologies are a result of developmental mechanisms which contain an innate degree of plasticity (Fabian 1989). Phenotypic variations can arise during ontogeny but there are 'buffering or compensatory' mechanisms which resist these variations (Fabian 1985). Balon (1989) felt that these 'buffering or regulatory mechanisms' and the 'unstable phases' of Fabian (1985) were representative of the properties of his 'homeorhetic steps' and 'thresholds', respectively.

Plasticity in the phenotype and the life-history style of Oreochromis mossambicus

Cichlid fishes are ideal animals for conducting research due to their prolific nature and their plasticity. Generally, they breed readily in captivity, thus giving the researcher ample specimens for short and long term investigations. Because O. mossambicus is easily bred and exhibits such a wide range of ecophenotypes and life-history traits, it was chosen for this project. The deposition of clutches of numerous eggs which can be fertilized by the same male, assures ample numbers of genetically similar offspring incubated under identical conditions. Although more than

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one spawning may be necessary from the same or different adult
fish, thus increasing error due to variation, this error is less
than when using combinations of several males and females over a
long period of time, as would be the case for daily spawners.

O. mossambicus exhibits a particularly high degree of plasticity,
both phenotypically and in its life-history style. Their natural
distribution ranges from southern Kenya, along the inland and
coastal regions of southeastern Africa and southward to the
eastern Cape, South Africa (Philippart & Ruwet 1982). They are
found in riverine, lacustrine and estuarine habitats ranging in
salinity from freshwater to hypersaline. They are capable of
tolerating temperatures between 11°C (Allanson *et al.* 1971) and
38.2°C (Allanson & Noble 1964), pH values from 5 to 10 (Swingle
1968) and salinities of 0 to 120 °/oo (Potts *et al.* 1967, Wallace
1975, Assem & Hanke 1979). They can be opportunistic feeders but
their preferred diet consists mainly of benthic algae, especially
diatoms, and detritus (Trewavas 1983). Like many other cichlids,
O. mossambicus exhibits a highly specialized mode of reproduction
and belongs to the reproductive guild of mouthbrooding bearers
without buccal feeding (Balon 1990). The males excavate bowl-
shaped nests in the substratum, often in arenas, to which they
attract females for courtship. After the female lays a batch of
eggs, the male slowly swims over the eggs while releasing sperm.
At the same time, the female takes the eggs and the emitted sperm
into her mouth where further fertilization occurs. This behaviour
is repeated several times until all the eggs have been spawned.
Mouthbrooding females may form schools in shallow, littoral areas
of lakes (Bruton & Boltt 1975). The eggs are constantly churned
in the female's buccal cavity and hatching occurs three to five
days after fertilization (Vaas & Hofstede 1952, Lombard 1962). The
time of first release from the female's mouth varies between 11 to
14 days and the female may recall the young into her mouth when a
threatening situation arises. Final release occurs after 14 to 22

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days (Vaas & Hofstede 1952, Bohrer 1953). Juvenile growth is usually rapid. The age at first maturity can be between three months and three years, and the life-span ranges from four years under unfavourable conditions to 12 years in more favorable habitats (James & Bruton 1991a). The minimum and maximum recorded adult weights are 15 to 30 g (Hecht & Zway 1984) and 3.2 kg (Bruton *et al.* 1982), respectively. O. mossambicus is a seasonal or continuous serial spawner depending on the conditions of their habitat. As with most fishes, fecundity is closely related to female size and body condition (Bagenal & Braum 1968) but can be influenced by environmental conditions. For example, Reznick & Endler (1982) found that guppies living in a habitat with high predation pressure on adults produced more and smaller offspring than two other populations with: 1) moderate predation, predominantly on juveniles, and 2) low predation on all age classes.

The ability of this species to survive in a wide range of habitats and to express diverse suites of characters in their life-history style is a reflection of their plasticity. Physico-chemical conditions such as temperature, depth, salinity, dissolved oxygen and turbidity, and biological factors such as food, density and predator pressure, can result in the manifestation of different ecophenotypes or life-history strategies. James & Bruton (1991a & b) compared the demography and life-history styles of populations of O. mossambicus from habitats differing in their biotic and abiotic conditions in the eastern Cape, South Africa. The one population expressed precocial characteristics of fast growth rates in both juveniles and adults, long life, delayed maturation at a high mass, low spawning frequency and relatively low fecundity. Phenotypically, these fish were deeper bodied and more robust than any of the other populations in the area. Ecologically favourable conditions allowed for rapid growth. The main contributing factor was the quantity and quality of available food. Also of

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importance was a greater number of suitable habitats for feeding, breeding and refuge, and more homothermal temperatures within the water column. The habitats where the populations of fish expressed precocial characteristics were considered to be more stable, less harsh environments than the other habitats investigated. Two other populations were characteristically altricial. In one case, their life-history characters included high juvenile mortality, early maturation, high reproductive output and short life-span. Although food was abundant, the variable physico-chemical factors caused this environment to be harsh. In the other altricial population, the food quality was good for juveniles which matured at an early age and small size but grew little afterwards. The steep underwater profile, low winter temperatures, abundant reeds in the shallow areas and non-cyclical variation in physico-chemical variables were all contributing factors to a harsh habitat.

The parent fish used in this thesis were obtained from the population that inhabited the stable, more predictable, less harsh environment (Mill Farm dam) investigated by James & Bruton (1991a & b) and expressed precocial life-history traits.

Hypothesis

The hypothesis being tested here is that in early ontogeny there are homeorhetic states of relative stability during which the necessary components synchronize their development before switching to another level of complexity. The switch occurs at an accelerated rate during periods of vulnerability. Internal and external influences determine the timing of these events and the trajectory followed thereafter. These influences do not occur in isolation but are the result of complex networks and interactions.

CHAPTER 2: MATERIALS AND METHODS

Collection site and procedure

All the fish used in this study were collected from Mill Farm Dam, 5 km outside Bathurst, Cape Province, Republic of South Africa (26° 54' E, 33° 33' S). This small impoundment is approximately 10 ha in area with an average depth of 2 m but ⁻ reaches a maximum depth of 4.6 m during periods of high water flow. The dam is filled through ground water seepage and surface run-off. It has a low profile and, due to a high incidence of cattle and waterfowl, the run-off water creates a meso-eutrophic water body and nutrient sump. The major plant species in the dam is <u>Potamogeton pectinatus</u>. This site was considered to be a stable habitat and the fish in this population exhibited the suites of characters associated with precocial ecophenotypes and life-history styles (James & Bruton 1991a & b).

All the specimens were collected in October and November 1987 and were obtained using a fleet of gillnets of varying mesh sizes. These nets were checked every quarter of an hour to prevent excessive stress and injury. The fish were transported live to the laboratory in large plastic barrels and placed in a holding tank in a controlled-temperature room.

Laboratory set-up

All the tanks described below were housed in a controlled-temperature room with a photoperiod of 14 h light, 10 h dark. Ambient temperature was usually only a few degrees below the desired water temperature of 25°C.

Brood stock tank: Newly caught fish were placed in a 1400 l tank in which the temperature varied between 20 and 22°C. Several tagged fish, which had adjusted to the conditions of captivity, were also present in the tank to aid in acclimatizing the wild fish to captivity. The tank was treated with acriflavine, a fungicide, for several days after each introduction of the new fish to prevent possible fungal infections due to the stress of handling. The tank was made of asbestos sheets with three panels of glass placed along the front. Gravel of varying coarseness was layered behind false walls of fiberglass at either end of the tank. Submersible pumps were placed on top of the gravel which recycled the water back into the main tank area through a plastic pipe over the top of the wall. Large airstones were placed at the inlet of the pumps to facilitate aeration and to prevent the layer of water above the gravel from stagnating.

The breeding tank was a 1400 l asbestos tank with viewing windows and false end walls as described above. Gravel was layered on top of a false bottom consisting of slitted, corrugated fibreglass sheets placed on top of brick supports. Submersible pumps were placed behind the walls with their outlet pipes extending into the main tank area through holes in the false wall panels (Figure 1a). Venturis attached to the pumps served to aerate the water in the main area while airstones kept the walled-off water from becoming stagnant. The total combined flow rate of both pumps was 1640 litres per hour. In order to prevent a break-down of the filtration system due to the nest-building behaviour of the fish, nylon screening was placed below the uppermost layer of gravel. A double-tubed, 2.5 m fluorescent light fixture was placed 0.77 m above the water surface of the tank. Three 200 watt aquarium heaters were placed in the tank and connected to an electronic relay box (custom made by Labotec Natal (Pty) Ltd.). Temperature control was maintained via a contact thermometer which was connected in series to the heaters through the relay box.

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The eggs, embryos and larvae were incubated in a 60 l glass aquarium. A simulated mouthbrooding action was obtained by connecting steep-sided separating funnels to the outflow of a Fluval 102 outside filter/pump (Figure 1b). The outlet of the funnels flowed through transparent plastic tubing into small plastic jars covered in fine netting which were suspended in the incubator tank. Within the filter, the water passed through a layer of gravel, charcoal and glass wool. The same heating system as described above was used except that only one heater was required. One small airstone was placed in the tank. In order to obtain a low light level, the tank was placed within an enclosure with access on one side only where a piece of black cloth was hung.

Laboratory procedures

Several attempts to induce successful spawning were made using various combinations of male:female ratios beginning in October 1987. In January 1988, a successful spawning was obtained using one male with six females and it was this ratio which was used throughout the remainder of the project. The initial water temperature was 23.5°C, and the heating system was enacted to maintain the tank at 25±0.5°C throughout the breeding observations. Spawning behaviour was closely observed and the time of the first egg deposition and the last sperm uptake by the female was recorded. Activation time (t = 0) was considered to be the midway point in time between these two activities and was used for ageing purposes for each clutch. The removal of the eggs from the female's mouth occurred at any time from immediately after spawning to up to 3 days, 16 hours after spawning (Table 1). The eggs were placed in the incubation funnels and kept at a constant temperature of 25±0.5°C.

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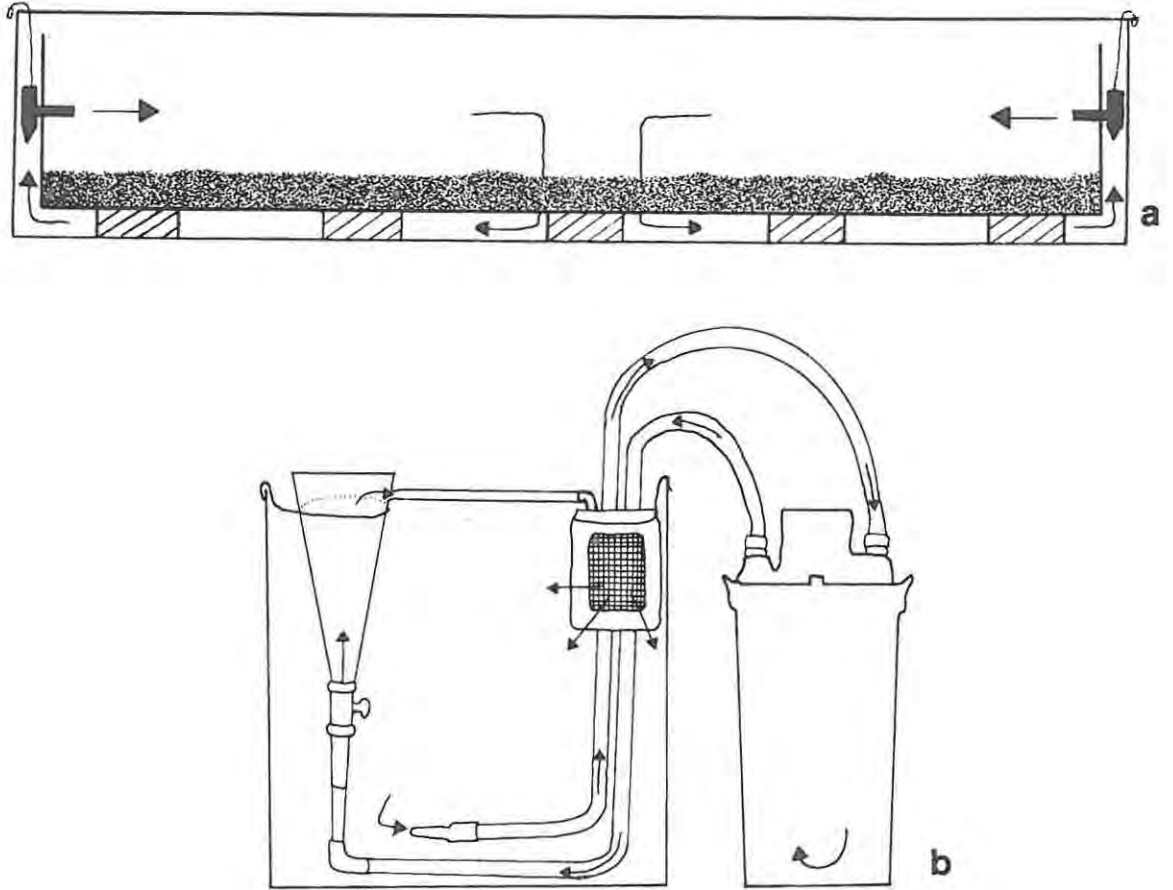
Figure 1 Side view of the breeding tank (a) and the incubation system (b). Arrows indicate the direction of the water flow.

The specimens used for this study came from six different spawnings of three females. Four of these spawnings occurred in January and February 1988 and the remaining two in November 1988. The description of the early ontogeny was derived from individuals from all the clutches. The coding of these batches was 3, 4, 4.2, 6, 6.2 and 6.3. The first numeral refers to the first spawning of a given female and the second numeral indicates subsequent spawnings of the same female (i.e. 6 is the first batch of female 6, whereas 6.2 was the second clutch). Although some information

was obtained from specimens of all six batches, some clutches contributed more than others. Offspring from the first clutch of female 6 contributed the main bulk of information. Clutches 4.2 and 6.3 were used predominantly to check for variation and first exogenous feeding. Clutch 4 was only used for the first seven samples and clutches 3 and 6.2 were used sparsely during the first five days.

Table 1 Removal times after activation of six clutches of _Oreochromis mossambicus_ eggs from the buccal cavities of the females.

Clutch number	Removal time
3	20 hours
4	immediately
4.2	1 day
6	14 hours
6.2	3 days, 6 hours
6.3	2 days

Many embryological studies concentrate on the development of a particular structure, organ or organ-system using scanning electron microscopic and histological techniques. Their purpose in doing so is often for identification and for comparisons between families, genera and species. My approach has been more holistic in nature and documents the overall development of the organism. I attempted to ascertain how the various tissues, organs and organ-systems interact, not only on a morphological or anatomical level, but also at a functional level. In order to accomplish this, it is necessary to use live and preserved specimens which are sampled

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at predetermined and frequent intervals. The methods of Balon (1985) are best suited to the purposes of this study and it is for this reason that I have conducted this project according to his procedures, as outlined below.

Samples were taken from the time of activation until juvenilization at varying time intervals depending on the level of development and the importance of the ontogenetic events expected to occur at a given time. Often more than one individual at a time was removed. The specimens were placed in a large depression slide and positioned under a Nikon SMZ-10 stereo-zoom microscope with a Microflex HFX-II photomicrographic attachment. Photomicrographs were taken and drawings were done using a drawing tube attachment. A fibre optic light source (Fi L151) was used for reflected light in order to avoid overheating of the specimens during the microscopic observations. Specimens were placed in vials for preservation and storage in a buffered formalin solution (1.8 g each of sodium phosphate monobasic and anhydrous sodium phosphate dibasic in 1 litre of 5% formalin).

Treatment of live specimens

The reason for the different removal times from the female's buccal cavity was due to high mortalities experienced when the eggs were removed too soon after spawning. Subsamples of eggs from four of the six batches were taken and weighed on a Sauter electronic, analytical balance accurate to 0.0001 g. The eggs were then placed in a drying oven set at 75°C. Dry and wet weights per egg were determined by dividing the total weight of the sample by the number of eggs in that sample.

A glass tube with a rubber bulb at its end was used to suck out the eggs and the embryos from the funnels for microscopic examination. As soon as cardiac contractions were noted, the

heartrate was determined by timing 10 or 20 beats with a stopwatch. When the activity of the embryos became such that the researcher's ability to do drawings or take photomicrographs was hampered, the specimens were anaesthetized with a 1 ppt solution of MS222 (tricaine methane sulfonate). A few drops were added to the depression slide after recording behavioural and morphological information such as eye, jaw and fin movements and heartrate.

The embryos were collected in the plastic jars at the end of the outflow tube in increasingly larger numbers as they became free-swimming and mobile. Fourteen days and 16 h after activation, the jar containing batch 6 embryos was removed from the darkened enclosure and placed in a tank with a photoperiod as described above. Eighteen hours later the clutch was divided into two jars and at the age of 19 d, 22 h, all individuals were placed in a cloth-meshed breeding basket (125 mm x 160 mm x 130 mm). Clutches 6.3 and 4.2 were removed from the incubator and placed in breeding baskets at the ages of 16 d and 14 d, 22 h, respectively. Notes were compiled on aspects of the behaviour of the embryos in their simulated environment, such as swimming ability and time of first exogenous feeding. Although the information obtained under these artificial conditions may not be a direct reflection of natural events, it does indicate the level of development, both morphologically and functionally, of the offspring at that point in its ontogeny.

Individuals from clutches 6, 6.3 and 4.2 were the only ones used for samples near or during the time of first exogenous feeding. Food was added to the incubating funnels in advance of the expected time of first exogenous feeding. At the age of 8 d, 20 h batch 6 individuals were fed brine shrimp nauplii (<u>Artemia salina</u>) once a day and checked for food in the gut during normal sampling. Clutch 3 and 4.2 individuals were monitored more closely and were

fed two or three times per day beginning from the ages of 8 d, 1 h and 7 d, 18 h respectively. Commercially produced fish embryo food (Tetra Min Baby Fish Food "E" and Liquifry No. 1) and live brine shrimp nauplii were added to the funnels and jars at regular intervals and the samples were removed periodically to check for food in the gut. A transparent plastic tray was placed at the bottom of the breeding baskets to prevent the nauplii from falling out. The embryos were fed twice a day from the age of 12 d onwards for all clutches.

Treatment of preserved specimens

Attempts were made to clear and stain unhatched specimens according to the techniques of Galat (1972), but with little success. The major problems encountered were related to the consistency of the yolk. In samples with the chorion intact, the stain was taken up by the egg envelope and did not penetrate through to the embryo. In dechorionated samples, the yolk stained and disintegrated into numerous, crumbly clumps and consequently damaged the area where the embryo was positioned. In some cases, it appeared that chemicals used to remove the chorion had damaged the embryo.

Yolk consistency was also a problem in attempts to peel the embryo off the yolk when the chorion was successfully removed. Because of the close association of the cells of the embryo and the yolk, large clumps of yolk material adhered to the embryo which was often broken into indistinguishable pieces.

Samples of newly hatched individuals which were stained with hematoxylin following Galat's (1972) techniques did not retain sufficient stain to reveal any useful information.

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In order to follow the development of the skeletal system, samples were cleared with trypsin and stained with alcian blue for cartilaginous mucopolysaccharides and with alizarin red-S for calcium phosphate. The procedure used was that of Potthoff (1984) with some slight modifications. Duration times in a particular compound or solution were adjusted to suit the specimens under study. Because alcian blue solutions have the potential to decalcify bone, some specimens were stained with alizarin red-S only. These single stained samples were compared to the double-stained ones to determine whether any decalcification had occurred. Once the age at which first calcification had been determined, younger specimens were stained with alcian blue solution only.

Compilation of data

Composites of the developmental illustrations were derived from slides, drawings and cleared and stained specimens. Additional information for the descriptions was obtained from observational notes.

It was considered that extensive allometric measurements were of little value. Problems obtaining accurate values due to poor visibility, orientation of the specimens, small sample size (often n=1) and subjective biases about points of measurement were considered to be too prone to error. Despite these reservations, some measurements were taken from drawings and slides of live specimens. A ruler was used to determine the distance between the eye margin and the otic capsule and to measure the horizontal and vertical axis of the eggs. Total embryonic/larval lengths were measured using a computerized digitizer. It was not possible to calculate the volume of the yolksac for two reasons; 1) as drawings and/or slides were used, only two dimensions were measurable, and 2) the size and shape of the yolksac changed over time which did

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not allow for an appropriate formula to be applied to determine volume. For these reasons, it was decided to determine the area of the yolksac from the drawings using a digitizer. These data were used as an indication of relative changes in rates, sizes and/or patterns of development and are not to be taken as absolute values for a given time or interval.

One-way analysis of variance and pairwise t-tests were conducted on yolk area between clutches 3, 4 and 6. Univariate and multivariate analysis were done on maximum and minimum yolk lengths over time, both within and between clutches 3, 4 and 6. In both of the above instances, data from samples of subsequent spawnings of the same female were combined. Pairwise comparisons of the means and the bivariate distribution of minimum and maximum egg length were done between all six clutches. A regression analysis was conducted on yolk area versus time and embryo length versus time assuming two regimes for each regression. Due to large scatter and variation of the first four points , in testing for the regimes of yolk area versus age, these points were omitted from the analysis. The level of significance was $p \leq 0.05$ in all cases.

The terminology of ontogenetic events follows that of Balon (1975). The embryonic period has been divided up into three phases; cleavage (C), embryonic (E) and free-swimming (F). The superscript numeral refers to the step within the phase and the second numeral is the accumulative step from the time of activation (e.g. $E^1 4$ refers to the first step in the embryonic phase and the fourth step since activation). The age of the specimens was denoted as days:hours after activation, and represents the beginning of sampling time when the specimen was removed from the incubator or the breeding baskets. Activation is used as defined by Balon (1985, pg.20). The age is also expressed in temperature units (TU = degree-days or temperature units) and was calculated by multiplying age (hours/24) by temperature (25^0C). Varied sources

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were consulted for the terminology of morphological and anatomical structures. When particular embryological terms were not available, structures were named after the corresponding adult structures. In cases of uncertainty about exactly what structures were present or what the proper anatomical terms were, I state what I assume them to be or I simply describe what is present without naming it. The main source of information used for naming of blood vessels was Balon (1985). Skeletal structures were named according to numerous sources including Balon (1985), Cunningham & Balon (1985) and Greenwood (pers com). Generalized explanations of embryological processes were drawn mainly from Balinsky (1981) and Cohen & Massey (1982).

For technical and logistical reasons, the question of individual variation has not been addressed sufficiently, although an attempt was made to touch upon this aspect. Occasionally several specimens at a particular sample time were removed and individuals from different batches of the same age were periodically incorporated in the sampling schedule. Ideally, if the ontogeny of several individuals could be followed from time of activation until juvenilization, a more accurate account of the developmental rates and patterns would have emerged and the issue of individual variation could have been assessed. However, the effects of removal from and return to the incubators during the sampling regime would add yet another bias to the results. More sophisticated equipment which would allow continual observations without disturbance to the animal, could help to solve this problem. Two other problems encountered were the subjectivity of the researcher in determining the important developmental boundaries and thresholds and, in the case of Oreochromis mossambicus, the nature of the eggs which limits visibility, especially prior to hatching.

CHAPTER 3: RESULTS

Throughout this chapter an attempt is made to describe the developmental events chronologically. In the initial stages this ordering of events was possible, but the increase in complexity of the organism over time rendered this approach inefficient and led to confusing, fragmented descriptions. When necessary, for the sake of clarity, the descriptions were, therefore, divided into groups of systems which were subsequently handled chronologically.

Steps in the early development of <u>Oreochromis mossambicus</u>

Embryonic period   00:00-11:00 (TU = 0 to 275)

Cleavage phase   00:00-01:11 (TU = 0 to 37)

The fertilized eggs had an ovoid shape with the vertical axis slightly longer (mean = 3.04±0.20 mm) than the horizontal axis (mean = 2.19±0.16 mm). The vegetable pole was marginally broader than the animal pole. Table 1 shows the mean dimensions of the eggs of different and combined batches. Clutch 6 had the smallest eggs and clutch 6.2 had the longest but not the widest eggs. Egg size differences between some of the clutches was significant and is discussed below in the 'variation' section. Clutch 4 and 6.3 were the lightest and the heaviest, respectively, of the four clutches weighed (Table 2). The large, yellow yolk was opaque with a uniform consistency. No obvious oil globules were present. The chorion was translucent and had a slightly dimpled texture. This made observations of cell number, size and orientation, as well as tissue and organ-system development, extremely difficult. The density of the yolk exacerbated this problem especially with regard to the movement of the blastodisc during epiboly.

Table 1 Mean maximum and minimum length of O. mossambicus eggs from different clutches (SD = standard deviation)

Batch number	Sample size (n)	Mean maximum length (mm)	SD	Mean minimum length (mm)	SD
3	12	3.09	0.15	2.20	0.09
4	7	3.10	0.08	2.14	0.03
4.2	3	3.03	0.02	2.18	0.06
6	12	2.80	0.19	2.02	0.13
6.2	10	3.22	0.11	2.32	0.11
6.3	4	3.14	0.03	2.40	0.05
Total	48	3.04	0.20	2.19	0.16

Table 2 Wet and dry weights of O. mossambicus eggs taken from clutches 3, 4, 4.2 and 6.3.

Batch number	Wet weight (g)	Dry weight (g)
3	0.0088	0.0041
4	0.0079	0.0032
4.2	0.0091	0.0052
6.3	0.0102	0.0058

$C^1$1 00:00-00:01 Bipolar differentiation, egg envelope hardening and the formation of the perivitelline space occurred in this step.

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One hour after activation (TU = 1), the cytoplasm had gathered at the animal pole forming a one-celled blastomere. The perivitelline space was visible only above the cytoplasm. The micropyle had a cone-shaped configuration (Figure 1a).

$C^2$2 00:01-00:22   This step began with the first cleavage, continued with germ ring and periblast formation and ended with the flattening of the blastodisc just prior to epibolic movement.

By age 00:02 (TU = 2), the first division of the cytoplasm had occurred. The perivitelline space above the blastodisc was larger and was also visible at some points along the yolk periphery (Figure 1b). The second division had been completed by age 00:03 (TU = 3) and occurred vertically. The distal margin of the blastodisc extended dorsally and was close to contacting the egg envelope (Figure 1c). At age 00:04 there were 8 cells of similar size, and division still appeared to be on the meridional plane (Figure 1d). By age 00:05 (TU = 5), there were 32 cells and horizontal division had taken place (Figure 1e). During the next two hours, cleavage continued such that the perivitelline space was filled by the still large-celled blastodisc of undetermined cell number. By age 00:08 (TU = 8), it was not only impossible to see what was happening within the egg in terms of its development but it was difficult to say whether the eggs were even viable. All the remaining eggs from this batch died and were discarded.

Specimens from another batch which were sampled at hourly intervals from age 00:15 to 00:17 (TU =15 to 18) consisted of flattened blastodiscs of innumerable cells of extremely small dimensions (approximately 0.023-0.028 mm in diameter). Some perivitelline space was visible both above and along the sides of the blastodisc. From age 00:18 (TU = 19) until the end of this step a transparent band, the periblast, was visible around

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the circumference of the yolk, just below the lip of the blastodisc (Figure 1e). Small cells along the periphery of this band were also visible.
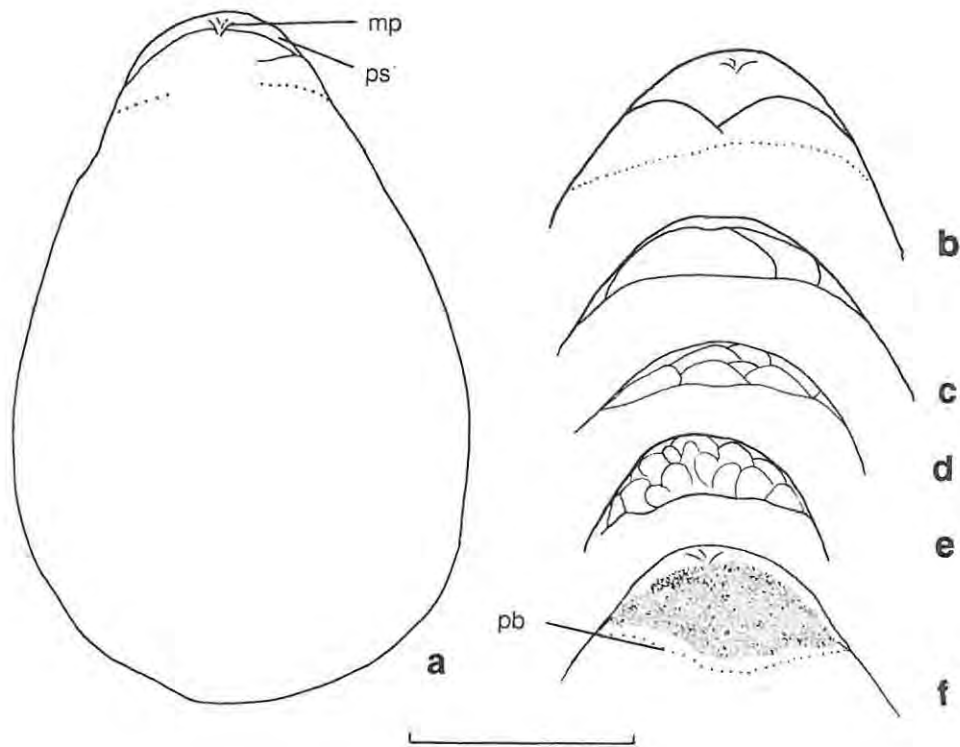
Figure 1 Lateral views of steps $C^1 1$ and $C^2 2$: a - age 00:01 (TU = 1), b - age 00:02 (TU = 2), c - age 00:03 (TU = 3), d - age 00:04 (TU = 4), e - age 00:05 (TU = 5), f - age 00:18 (TU = 19) (mp = micropyle, pb = periblast, ps = perivitelline space). The total number of blastomeres is not visible in all the drawings due to egg orientation. Scale = 1.0 mm.

$C^3 3$ 00:22-01:11   This step began with epiboly. The embryonic shield formed and differentiation at its anterior end marked the end of this step.

By age 00:22 (TU = 23), individual cells were no longer distinguishable and the blastodisc was greatly flattened (Figure 2). The lip of the blastodisc extended approximately 10-15% down

over the yolk. This signified that epiboly had begun. A slightly skewed blastodisc was observed in some of the specimens during this step which indicated that the movement of the leading edge of the germ ring over the yolk is not uniform and that cells were aggregating to form the embryonic shield. By age 01:11 (TU = 37), a ridge could be seen along one side of the blastodisc which extended from just below the apex of the yolk to almost the equator and was in contact with the envelope wall along its dorsal aspect. Slight vertical and lateral swelling in the anterior region of the embryonic shield was an indication that neurulation had begun. Although no distinguishable structures were visible, the configuration of these groups of cells implied that some differentiation was occurring in the region of the brain and the otic vesicles. These events not only mark the end of a step but they precede the shift into the embryonic phase.
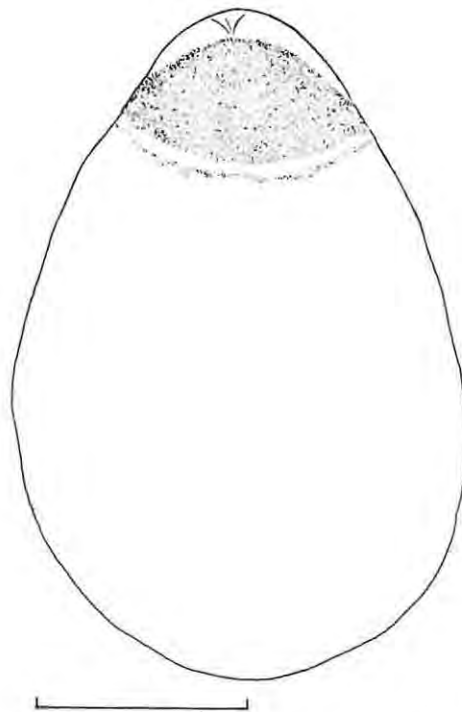

Figure 2 Lateral view of step $C^3 3$, age 00:22 (TU = 23) showing the flattening of the blastodisc as epibolic movements begin. Scale = 1.0 mm.

Phase summary: Throughout this phase, there were no external

environmental changes but internal changes occurred due to underlying cellular processes and interactions. In most teleosts, the eggs consist of a large yolk which is concentrated at the vegetable pole, and a thin layer of cytoplasm around the periphery (most of the egg cytoplasm is found in this outer layer). Close to the egg surface are cortical granules which are discharged when a sperm successfully attaches to the outer egg envelope. Water then rapidly permeates through the egg envelope, thus forming the perivitelline space. The egg envelope then hardens and most of the cytoplasm gathers at the animal pole forming a cytoplasmic 'cap.' Fertilization of the egg is the first of many inductive embryonic acts and initiates cleavage, the first major developmental process (Hall 1983). According to embryological textbooks, there is a steady increase in nuclear material but no growth occurs during cleavage (Balinsky 1981, Cohen & Massey 1982). The combined masses of the blastomeres are only slightly less than the mass of the initial parent cell due to some energy utilization and the production of waste products. The blastodisc consists of two general types of cells. A cohesive layer one cell thick, named the enveloping layer, covers the outer surface of the blastodisc. These cells participate in epiboly but do not contribute to embryonic tissue development. Beneath and within the enveloping layer are the deep cells which are involved in the morphogenetic movements that lead to the formation of the germ ring, the embryonic shield and, eventually, the embryo. A synctial layer, the periblast, which arises by incomplete division of peripheral and basal cytoplasm, separates the enveloping layer and the deep cells from the fluid yolk. The nuclei from the incomplete cytoplasmic divisions continue to divide and reside in the cytoplasm, thus forming the synctium. The periblast, like the enveloping layer, does not contribute directly to the development of the embryo; it aids in the breakdown of the yolk which is utilized by the growing embryo and results in the formation of the subgerminal cavity. The periblast undergoes epiboly and may serve as the substratum for the descending blastodisc during epiboly (Trinkaus 1969). In this study, at the end of the second step blastulation was complete

and the three germinal layers had formed in the blastodisc. During epiboly, the blastodisc flattens and slowly spreads over the yolk until it completely encloses it by the closure of the yolk plug. Simultaneously, the advancing edge of the blastodisc thickens to form the germ ring and the cells of the germ ring converge mid-dorsally to form the embryonic shield. Morphogenetic movements translocate and arrange cells and groups of cells resulting in the formation of endoderm, mesoderm and ectoderm.

The steps in this phase can be equated with the concepts of potential and kinetic energy; although there is not necessarily a change in energetics, there is a change from the potential to develop into another quantitative and/or qualitative state and the realization of that state. The unfertilized egg is a gamete with the potential to become a zygote, the former being a relatively inactive, stable state while the latter is an active, dynamic state. (I must emphasize here that there is in essence no inactive or non-dynamic, static state but rather that all entities, regardless of their level of complexity, are dynamic and in a state of continual change and interaction. Thus the use of the expression, 'relatively inactive, stable state'). Once activation has occurred, the realization of that potential is obtained through a visible increase of activity, i.e. breakdown of cortical granules, water permeating through and hardening of the egg envelope, cytoplasmic movements, etc. The formation of the cytoplasmic 'cap' can be considered the end-point of this active state, by which time a level of relative stability has been reached. In the second step, cleavage involves another dynamic and active interval. Once the blastula contains a certain number of cells, it flattens and the third step begins. Epibolic movements commence, followed shortly by morphogenetic movements, thus, signifying another apparent increase in activity. Throughout this third step there are quantitative and qualitative changes in the cells.

Embryonic phase   01:11-04:16 (TU = 37 to 117)

E^14 01:11-02:12   At the beginning of this step neurulation began as slight swellings in the anterior region of the embryonic shield and continued until distinguishable fore-, mid- and hindbrain components and optic vesicles were visible. At least 17 pairs of somites, four neuromeres and the presumptive pericardial cavity were formed. Body and yolksac melanophores appeared.

At the beginning of this step, the embryonic shield was obvious as a thickening along the dorso-lateral margins of the yolk and the presumptive pericardial cavity was visible as a thin, transparent chamber extending along the top of the yolk (see Figure 3a). The leading edge of the periblast had reached the yolk equator and the tail mound of the presumptive embryo had begun to form. Over the next nine hours the length of the embryonic shield ranged between 1.5 to 1.7 mm with its posterior end slightly above the margin of the descending periblast. An excised specimen at age 01:19 (TU = 45) consisted of aggregations of small cells of equal size which gave shape to the following structures but did not actually form any clear-cut divisions or boundaries as they appeared through the chorion. A fine line along the body axis indicated that some notochord development had begun. It appeared that the presumptive tissues and organs had begun differentiating mainly as a function of cell-to-cell interactions, thus giving them their shape but not necessarily forming divisions between the structures. Hints of somite formation were also noted in this specimen as simple cell aggregations without clear-cut definition. There may be another step at the age when the first few somites were formed but poor visibility made it impossible to establish exactly when this occurred. Only after a certain number of somites were developed was it possible to recognize them as definite structures.
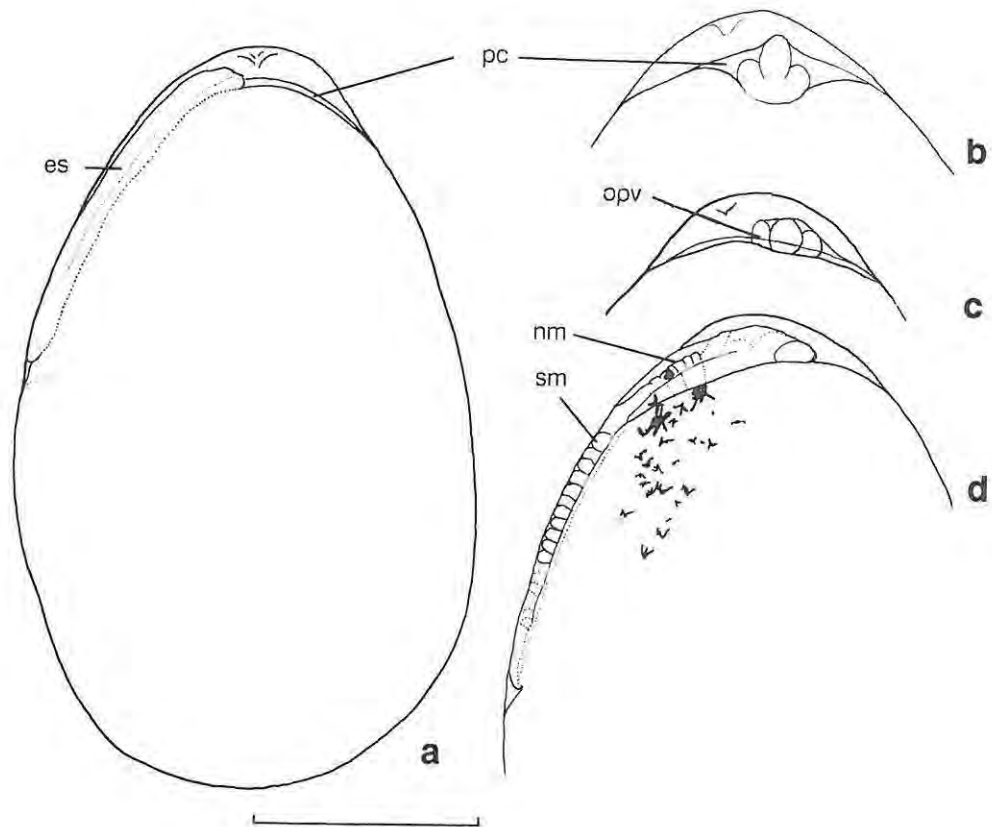
Figure 3 Step E¹4: a - lateral view at age 01:13 (TU =39), b - frontal view at age 02:00 (TU =50) showing the lack of differentiation of the optic vesicles, c - frontal view with slight rotation at age 02:00 showing optic vesicles in a more advanced stage than in b, d - right lateral view at age 02:03 (TU = 53) (es = embryonic shield, nm = neuromeres, opv = optic vesicles, pc = presumptive pericardial cavity, sm = somites). Scale = 1.0 mm.

Definite somites were visible from age 01:20 (TU = 46) until the end of this step. Poor visibility made it impossible to count exactly how many somites were present. However, the initial counts were 10 to 13 pairs with at least 14 to 17 pairs by the end of the step. The anterior somites were clearly defined but

the postanal ones were often indistinguishable as separate units because they were not as yet fully developed (Figure 3d).

The first appearance of pigments occurred at age 01:21 (TU = 47). There were two dark spots in the body tissue just posterior to and above the presumptive optic vesicles and pale, fibrous melanophores were present laterally on the yolksac near the head region. At age 02:00 (TU = 50), the optic vesicles began to form and appeared as lateral outpockets from the slightly differentiating fore- and midbrain (Figures 3b & c). In Figure 3c, the optic vesicles appear to be distinct from the presumptive brain but by rotating the egg slightly it can be seen that the level of development was less advanced than would be expected (Figure 3b).

The development of the presumptive pericardial cavity progressed throughout this step until it extended posteriorly along the body axis to the hindbrain where it then dipped ventrally towards the yolk. Dorsally it extended to about the middle of the optic vesicle. By age 02:03 (TU = 53) the constrictions in the brain made the prosencephalon (forebrain), the mesencephalon (midbrain) and the rhombencephalon (hindbrain) distinguishable from one another (Figure 3d). The optic vesicles were clearly separate from the brain. In the hindbrain some differentiation was occurring and four to five neuromeres were forming. The head extended farther forward over the dorsal surface of the yolk and was almost in contact with the egg envelope. The tail did not appear to be free of the yolksac. The yolk plug was not closed and about 66% of the yolk was covered by the migrating enveloping cells. Body pigmentation had extended slightly along the body axis at the yolksac junction behind the head. The yolksac pigmentation extended posteriorly and ventrally and covered a larger area. Aggregations of melanophores formed dark, dense regions on the dorso-lateral regions of the yolksac.

The mean total length of embryos between the ages of 01:20 (TU = 46) and 02:03 (TU =53) was 1.9 mm. During the entire step

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there was a gradual increase in embryo length of 0.5 mm and little change in yolk area.

Step summary: Tissue formation resulting from cellular differentiation and interactions had gradually established the basic body form and components. Underlying internal changes and processes were evident in the structures present. The function of the tissues is to supply the substratum for organ development in the subsequent steps.

In some animals, neurulation and organogenesis can begin before the closure of the yolk plug. This is the case with O. mossambicus.   During neurulation, the neural plate is formed along the embryonic shield and narrows gradually while sinking deeper into the underlying tissue.   During gastrulation and neurulation, another set of embryonic inductive interactions are initiated (Hall 1983).   For example, interactions between presumptive notochordal tissue and presumptive ectodermal tissue will induce neural differentiation.   Ectoderm which is not in contact with notochordal tissue will form epidermal ectoderm.

$E^2 5$ 02:12-03:00   This step began with the first heartbeat. Other events included the formation of the eye lenses, the heart-tube, the otic capsules and otoliths, first muscle contraction, further brain differentiation, elongation and separation of the tail, and anastomisation of blood islands and the anterior vitelline veins.

The first heartbeats were recorded from two specimens as 86 and 98 beats min^{-1} at age 02:12 (TU = 63) but no blood flow was detected.   Lenses had formed over the optic capsules. The mesencephalon formed the highest point of the head and had developed a small chamber in its central area (the optic ventricle).   The cerebellum appeared as a narrow, vertical projection immediately behind the midbrain, indicating that the

differentiation of the hindbrain into its two major components
(the cerebellum and the medulla oblongata) had begun (Figures 4a
& b). Although no body pigmentation was noted, the
configuration of the melanophores on the yolksac became stellate
in addition to circular. Body movements were first recorded at
age 02:16.

At age 02:20 (TU = 71), the tail was free from the yolksac and
active muscular contractions occurred. The heart was a
thinly-walled tube within the pericardial cavity and extended
from just anterior to the presumptive snout to the posterior
margin of the eye (Figure 4b). A few blood cells at a time could
be seen entering the heart-tube from a wide, pinkish region on
the yolksac which served as a collection area for blood cells of
the anastomosing respiratory vitelline plexus (Figure 4b).
Although some haemoglobin was present in the blood, the
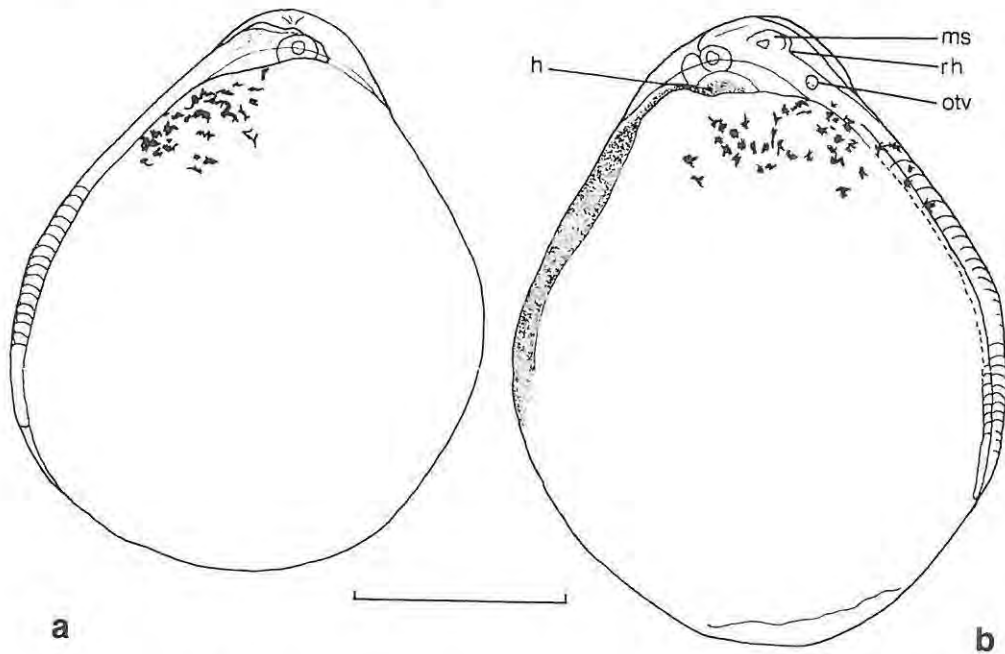

Figure 4 Step $E^2 5$: a - right lateral view at age 02:12 (TU =
63), b - left lateral view at age 02:20 (TU = 71), the stippled
area on the left of the yolksac shows an area which contained
blood cells in which there was no blood movement (h = heart-tube,
ms = mesencephalon, otv = otic vesicle, rh = rhombencephalon).
Scale = 1.0 mm.

quantities were not sufficient for detailed observations of vascular development. Most of the circulatory system was still undetectable except in areas of high blood concentration or extremely strong flow. Blood flow movement was seen along the pathway of the anterior vitelline veins and at the junction of the preanal plexus and the yolksac, but no distinct vessels were visible. There was no visible blood flow in the body but some vessel development must have occurred in the cardinal vein and the preanal plexus as evidenced by the presence of the above mentioned yolksac circulation. There were two cavernous areas at the ventral pole of the yolksac which presumably served as an area for blood formation and collection. Various indentations and channels were forming on the yolksac surface but they were not distinguishable as distinct entities. These isolated structures were the sites of blood islands and anastomosing vitelline vessels. The fourth ventricle of the brain was enclosed dorsally by a thin membrane above the medulla oblongata, and hemispherical division of the prosencephalon, mesencephalon and the cerebellum had occurred. Otic capsules were first seen at this time and were already well developed. They appeared as a closed ring and two otoliths were present on each side. The distance from the posterior margin of the eye to the anterior margin of the otic capsule was 0.31 and 0.43 mm in two individuals. The visceral cavity was visible between the body proper and the yolksac from posterior of the otic capsule to the presumptive anus. Melanophores were present along and perpendicular to the body axis in the region of the first five pairs of somites (Figure 4b). Somites extended almost to the tip of the elongated tail. Pigment cells were present above the visceral cavity and further ventral migration of melanophores had occurred on the yolksac. One pigment spot was seen on the envelope itself which was very delicate and easily burst during handling.

Throughout this step, the heart rate was very erratic, ranging from 86-151 beats min $^{-1}$. At age 02:20 (TU = 71), heart contractions of four individuals from one clutch ranged from 96-126 beats min $^{-1}$. Another three individuals of the same age from

a different clutch had a much higher rate between 143-151 beats min $^{-1}$.

The mean embryo length of three specimens was 2.9 mm. There was a substantial increase (0.7 mm) in length between the last specimen of the former step and the first individual in this step. There was no growth and very little change in yolksac area within this step.

Step summary: Rudimentary organs have developed and some level of functionality has been reached, especially in neural, respiratory and circulatory structures. Morphological features such as a larger, more differentiated brain, and activity such as muscle contractions were obvious, visible changes at a high level of resolution. Although underlying physiological processes were not investigated in order to assess functional completion, visible activity indicated that some level of operation had been reached. A basic communication system (the brain and parasympathetic nervous system) and primitive transport and gaseous exchange systems (vitelline, finfold and body vascularization) have been established and provide the fundamental building blocks for further development.

$E^3 6$ 03:00-03:20   Strong body circulation became evident at the beginning of this step. Numerous head vessels and the ventral median finfold respiratory plexus developed. Eye pigmentation began, the heart-tube started differentiating, the posterior vitelline respiratory plexus branched and some haemoglobin was produced. In the last few hours hatching began and the posterior tip of the notochord was flexed dorsally.

Further development in the otic capsules was noted by the presence of an additional inner ring that was forming ventrally and along the sides but not yet completed dorsally. The mean

distance between the eye and otic capsule margins was 0.27 mm (n = 6, range = 0.20-0.32 mm). Yolksac pigmentation became denser and in some areas the melanophores aggregated such that individual cells were not distinguishable. Pigmentation extended more ventrally on the yolksac surface, forming a band-like appearance. Changes in body pigmentation were minimal with a slight increase of cell proliferation along the body axis and above the visceral cavity. Darkening around the periphery of the optic capsules and along the circumference of the lenses was first noted half-way into this step. The mesencephalon extended more posteriorly and elongation of the prosencephalon anteriorly made the distinction between these two brain components more striking. The optic ventricle became tear-drop shaped and obvious, in part due to its transparency. Lobular development in the mesencephalon gave it the appearance of consisting of three separate components and there were six neuromeres in the hindbrain. At age 03:12 (TU = 88) the egg envelope had a cloudy and ragged appearance and at age 03:15 (TU = 90) hatching began (Figure 5c). When several individuals were removed at a given time, some were still contained inside the envelope, some had either their tail and/or head sticking out and others were completely free. In the hatched individuals it could be seen that flexion of the notochord had begun and the spinal column extended posteriorly beyond the notochord tip. The gastro-intestinal tract was a simple thick-walled tube with a fine central line but no lumen. It protruded slightly below the ventral median finfold, effectively separating it into a pre- and postanal component (Figure 5c).

Major events occurred in the development of the respiratory and circulatory systems. Constrictions and a slight left to right twist in the heart-tube was an indication that chamber development was underway. The circulation of blood through the body had begun in the previous step but was more pronounced at the beginning of this step as a strong flow in the dorsal artery. Within four hours, flow in vessels of the head region and the preanal plexus was also noted. The anterior vitelline veins

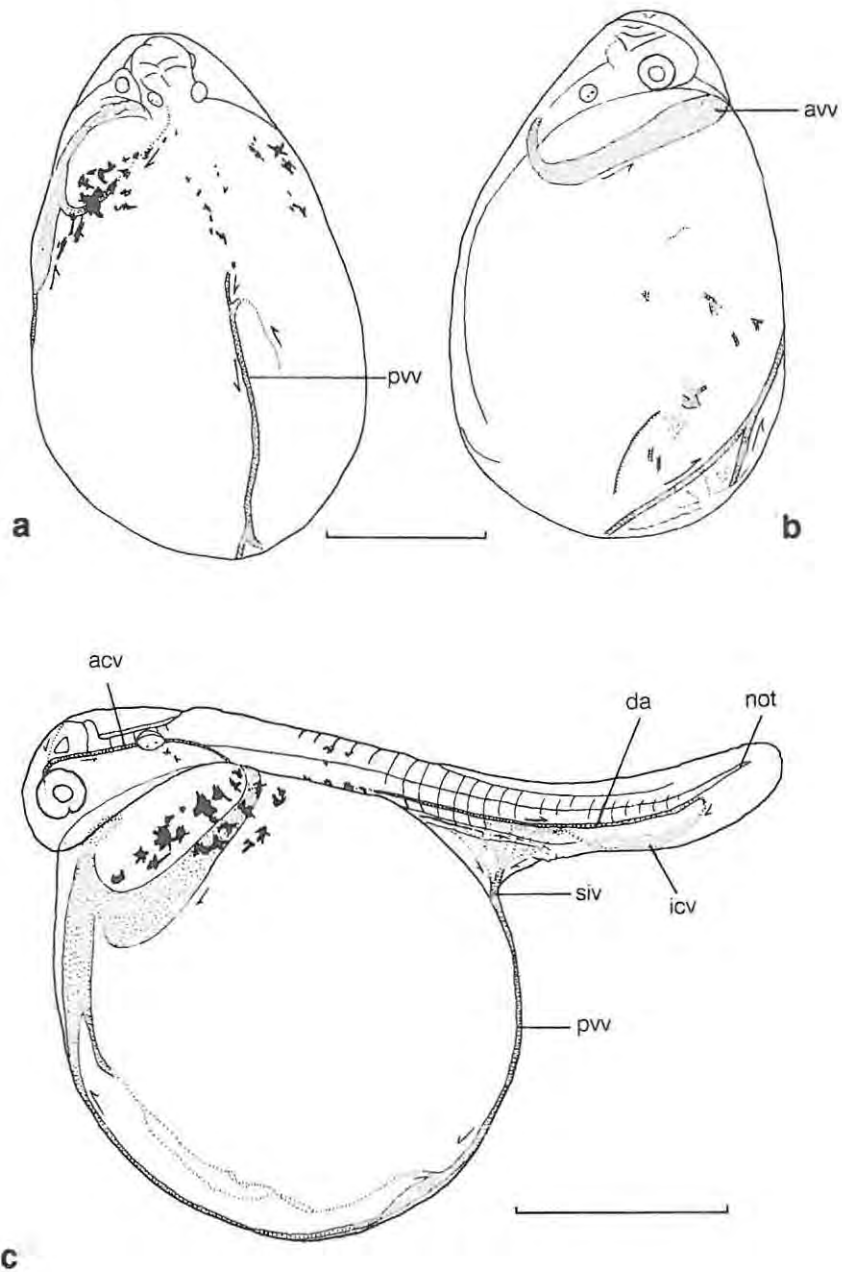**Figure 5** Step E³6: a & b - dorsal and right lateral view at age 03:10 (TU = 85) showing the early stages of development of the vitelline respiratory plexus, c - left lateral view at age 03:15 (TU = 90) of a newly hatched individual (acv = anterior cardinal vein, avv = anterior vitelline vein, da = dorsal artery, icv = inferior caudal vein, not = notochord, pvv = posterior vitelline vein, siv = subintestinal vein). Scales = 1.0 mm.

followed an arc-shaped path across the dorso-lateral surface of the yolksac. Blood from the preanal plexus collected in the subintestinal vein which then entered to the left of the yolksac into the posterior vitelline vein (see Figures 5a & c). A cavernous area on the ventrum of the yolksac served as a collection site for the blood from the posterior vitelline vein which, early in this step, did not appear to be branched. At age 03:10 (TU = 85), the posterior vitelline vein branched before the ventral, cavernous regions of the yolksac and branching vitelline vessels continued flowing dorsally from there towards the heart-tube (Figures 5a & b). Blood islands and anastomosing veins were more evident. The heart was slightly S-shaped and was beginning to tuck under the head. By the end of this step there was extensive head and finfold circulation. Blood flowing dorsally between the forebrain and the midbrain joined with the ventral flow from a vessel behind the eye. From there, a common vessel followed along the base of the mesencephalon and emptied into the anterior cardinal vein which continued posteriorly behind the otic vesicle before bending ventrally to enter the anterior vitelline vein (Figure 5c). The postanal plexus was fed by the dorsal artery which formed a single, caudal loop. The major vessel of this network, the inferior caudal vein, followed the distal margin of the postanal finfold, curved dorsally and rested below the dorsal artery where it fanned-out into the preanal plexus. Most of the flow between the two ventral finfolds was through the inferior caudal vein which collected blood from most of the minor vessels of the postanal plexus. There were a few minor veins forming a junction between the two finfold networks but they crossed over at a more posterior point than that of the inferior caudal vein. The preanal plexus was a complex network of fine vessels which flowed into the subintestinal vein which then emptied into the posterior vitelline vein. The posterior vitelline vein branched at the ventral pole of the yolksac into several smaller vessels to form a complex network which continued in a dorso-anterior direction over the surface of the yolksac. The blood from these vessels converged to form a collective, sheet-like flow prior to entering

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the heart-tube.   The vitelline plexus was difficult to see in detail except in the large vessels discussed above.

As in the previous step, the heartrate was inconsistent between samples of varying ages and of different parentage.   No pattern emerged regarding a relationship between heartrate and parentage. The first samples of this step had values of 124, 125 and 126 beats min^{-1} from three individuals of the same batch while an embryo from another batch of the same age had a heartrate of 143 beats min^{-1}.   In the last sample of this step a markedly higher heartrate was recorded from three individuals (mean = 197 beats min^{-1}).   The range of heartrates throughout this step was 90 to 205 beats min^{-1}; the lowest value was taken from a specimen aged 03:09 that appeared retarded in its development.

The length of the only embryo measured during this step was 3.4 mm.   There was a slight increase in length (0.5 mm) and no change in yolk area from the previous step.

Step summary: The major emphasis was on the establishment of simple circulatory and respiratory systems, especially in the temporary embryonic respiratory organs.   Functionally, these two systems allow for rapid development in other organs and organ-systems and meet the increasing oxygen demands that result from increasing physiological complexity.   There was a marked increase in activity, especially in tail movement, throughout this step which may have been due to a decrease in oxygen availability within the egg envelope.

E^{4}7   03:20-04:16   The development of the hepatic vitelline network, the branchial arteries and arterial flow into the preanal plexus began. Also of importance was the first appearance of the pectoral fin anlage. Almost all individuals had hatched by the end of this step.

Hatching continued throughout this step and the level of emersion out of the egg envelope varied between and within samples. Few notable changes were observed in all but the circulatory and respiratory systems. Pigmentation began to extend from the periphery of the optic capsules and the lenses to include the overall surface of the eye, giving it a peppery appearance. The mean distance between the otic capsule and the eye was 0.18 mm (n = 4, range = 0.15-0.22 mm). The pectoral fin anlage was apparent at the onset of this step as a small mound of tissue posterior to the otic capsule above the visceral cavity (Fig 6a). By the end of this step, it had developed into a semi-circular structure which extended dorsally to the middle of the notochord.

In the head, several vessels had formed loops in the brain region. Between the lateral hemispheres of the mid- and hindbrain an artery extended dorsally and proceeded posteriorly along the top of the brain for a short distance before bending ventrally behind the cerebellum (Figure 6b). The convergence of the right and left branches of the dorsal aorta into the dorsal artery was visible. In a small vessel dorso-anterior to the above junction, blood flowed anteriorly then formed a loop and continued in a posterior direction. In one specimen, an artery was noted above the notochord which extended posteriorly to mid-body. From that point a fine vessel flowed ventro-laterally. Early in this step some very fine vessels were noted in the area of the four gill pouches (Figure 6b). By age 04:09 (TU = 109), branchial arteries, which curved in a dorso-posterior direction, were noted in three of the four simple gill pouches. A fourth artery was noted between the branchial arteries and the eye which curved in a dorso-anterior direction. In all four of these arteries the blood pulsed strongly in a dorsal direction.

In the ventral finfold plexus there was an increase in the complexity of the finer vessels of the network and the major vessels became broader due to increased blood flow. Ventral to
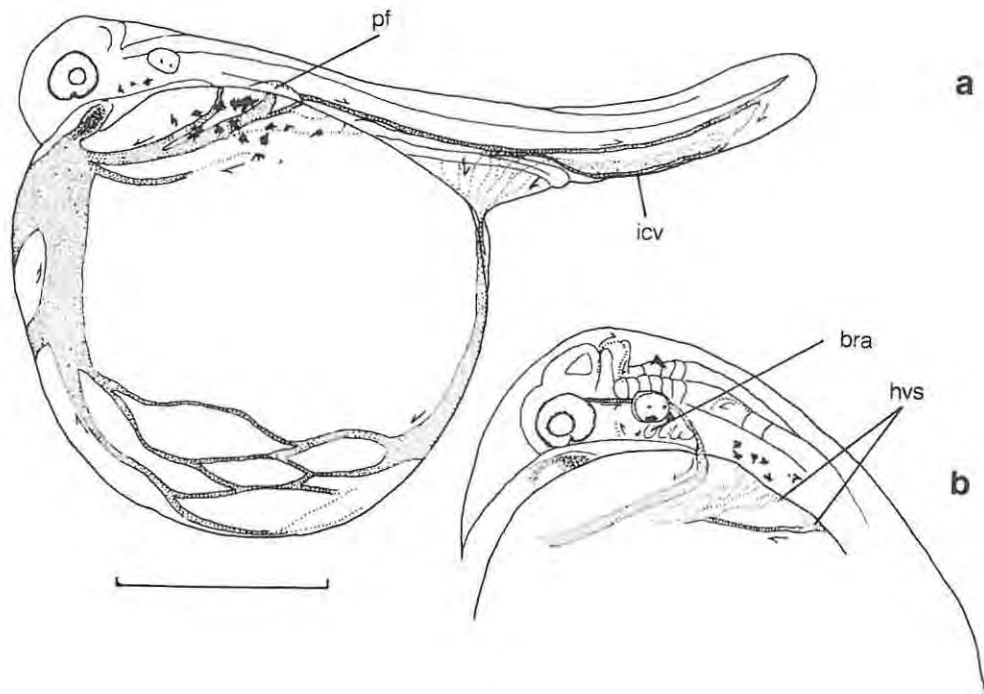
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Figure 6 Step E^47: a - lateral view at age 03:20 (TU = 96), b-lateral view at age 03:22 (TU =98) showing head, hepatic and branchial circulatory development (bra = branchial arteries, hvs = hepatic vitelline system, icv = inferior caudal vein, pf = pectoral fin). Scale = 1.0 mm.

the dorsal artery, an artery fanned-out into the preanal plexus, but the major contribution to the network was from the postanal veins. The origin of this arterial supply of blood was not clear, but judging by its position behind the yolksac, it was assumed to be from the coeliac-mesenteric artery.

Several veins on the upper left side of the yolksac at the beginning of this step signified the start of the hepatic vitelline system (Figures 6a & b). Several small veins and one major vein left the body cavity posterior to the anterior cardinal vein and ventral to the pectoral mound. Some of the smaller veins joined the anterior vitelline vein while others formed junctions with the major hepatic vein. In one specimen the veins entered to the right of the body. Throughout this

step, the hepatic vitelline network became increasingly more complex. Eventually, all specimens had venous networks entering the yolksac on both sides of the body. The pattern of the vessels was not symmetrical and showed variation amongst the individuals. In the majority of the cases, the network on the left side consisted of numerous fine veins, which through their branching and regrouping, formed an intricate pattern. Usually on the right side, the veins were broader but less numerous, which resulted in a more open pattern. As the complexity of the dorso-lateral vitelline veins increased, it was assumed that several blood vessels associated with the gastro-intestinal system were contributing to the network (such as the hepatic and mesenteric veins), but it was not possible to identify specific vessels. The posterior vitelline vein was a single vessel which branched into a complex network of veins on the ventrum of the yolksac. From this network one major vitelline vein continued anteriorly and was fed by several smaller veins. The anterior vitelline veins were also branched and formed their own networks. Blood from five major vessels fed by the networks described above (right and left anterior veins, right and left dorsolateral veins and the main vein of the subintestinal plexus), converged to form a sheet-like flow into the sinus venosus (Figures 7a-c & 8). The lateral areas of the yolksac appeared devoid of any flow or vessels but blood islands were visible. In some of the areas where blood accumulated, the yolksac had a cavernous appearance and was sunken in.

The mean total length of four specimens was 4.1 mm with a range from 3.8 to 4.4 mm. This indicates slight growth both within this step and between this and the previous step. There was no change in yolksac area.

The heartrate from the first sample of this step (175 beats min^{-1}) dropped markedly from that recorded for the last sample of the previous step (mean of 197 beats min^{-1}), increased slightly then decreased again in the last sample to a mean of 145 beats min^{-1}.

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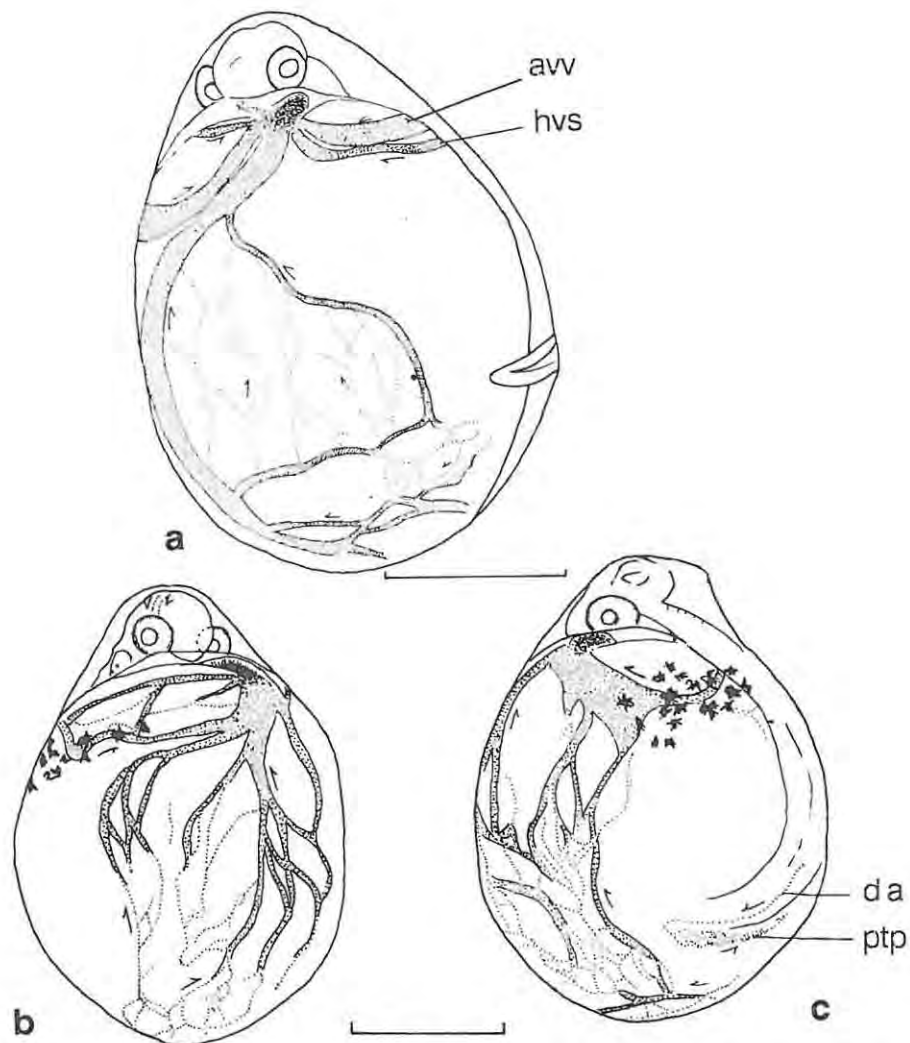
Figure 7 Step E⁴7: Frontal and lateral views of unhatched individuals showing the vitelline plexus: a – age 03:22 (TU = 98), b & c – age 04:02 (TU = 102) (avv = anterior vitelline vein, da = dorsal artery, hvs = hepatic vitelline system, ptp = postanal plexus). Scales = 1.0 mm.

Step and phase summary: Further complexity in the caenogenetic structures was the main morphological feature of this step. The formation of some basic structures of adult respiratory organs had begun, i.e. branchial arteries, but their ability to function in gaseous exchange was probably limited. The temporary vitelline and finfold respiratory plexi had developed and served to meet the increasing oxygen demands of the embryo. The digestive and sensory organs (except for the eyes) were simple but the brain and nervous system were well developed.

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Figure 8 Step E⁴7: Top - newly hatched individual at age 04:05 (TU = 105); Bottom - right lateral (left), dorsal (top) and right frontal (bottom) views of unhatched individuals at age 04:09 (TU =109) showing the vitelline respiratory plexus. Scales = 1.0 mm.

Growth throughout the embryonic phase occurred at a gradual pace (Figure 9a). The yolksac area had decreased slightly (Figure 9b). The overall trend in cardiac contractions was an increase in beats/min (Figure 10). Heartrate decreased at the end of this step and remained consistent into the initial stages of the next step. This plateau in heartrate encompassed the boundary and threshold between the embryonic and free-embryonic phases.

Because hatching began in the previous step and continued throughout this step and into the next one, the entire interval could be considered a transitory period rather than an abrupt and obvious leap. The exact timing of this threshold cannot be defined with precision because of individual variation and other factors that were not investigated in this study.

This was the first occurrence of external changes for the embryos. The internal environment of the incubator, and under natural conditions within the female's buccal cavity, remains the same but the embryos are subjected to a different oxygen regime outside the egg envelope. Release from the confines of the egg envelope allows for increased tail movement and oxygen uptake. This is reflected in the properties of the temporary respiratory structures. The individual has undergone gradual morphological and physiological development under stable, predictable conditions but the change in trophic features has been rapid. This change in conditions places the embryos at risk making them more vulnerable than in their previous situation.

The kinetic/potential energy analogy presented earlier is less applicable to step boundaries within the embryonic phase than to boundaries between the phases. At the end of the cleavage phase, the primordial germ layers are present and the potential to differentiate into specific cell types exists. The realization of that potential begins with neurulation and organogenesis which involves an increase in activity. In the first step of the embryonic phase the gradual morphological activity results in the establishment of a basic body form which has the potential to

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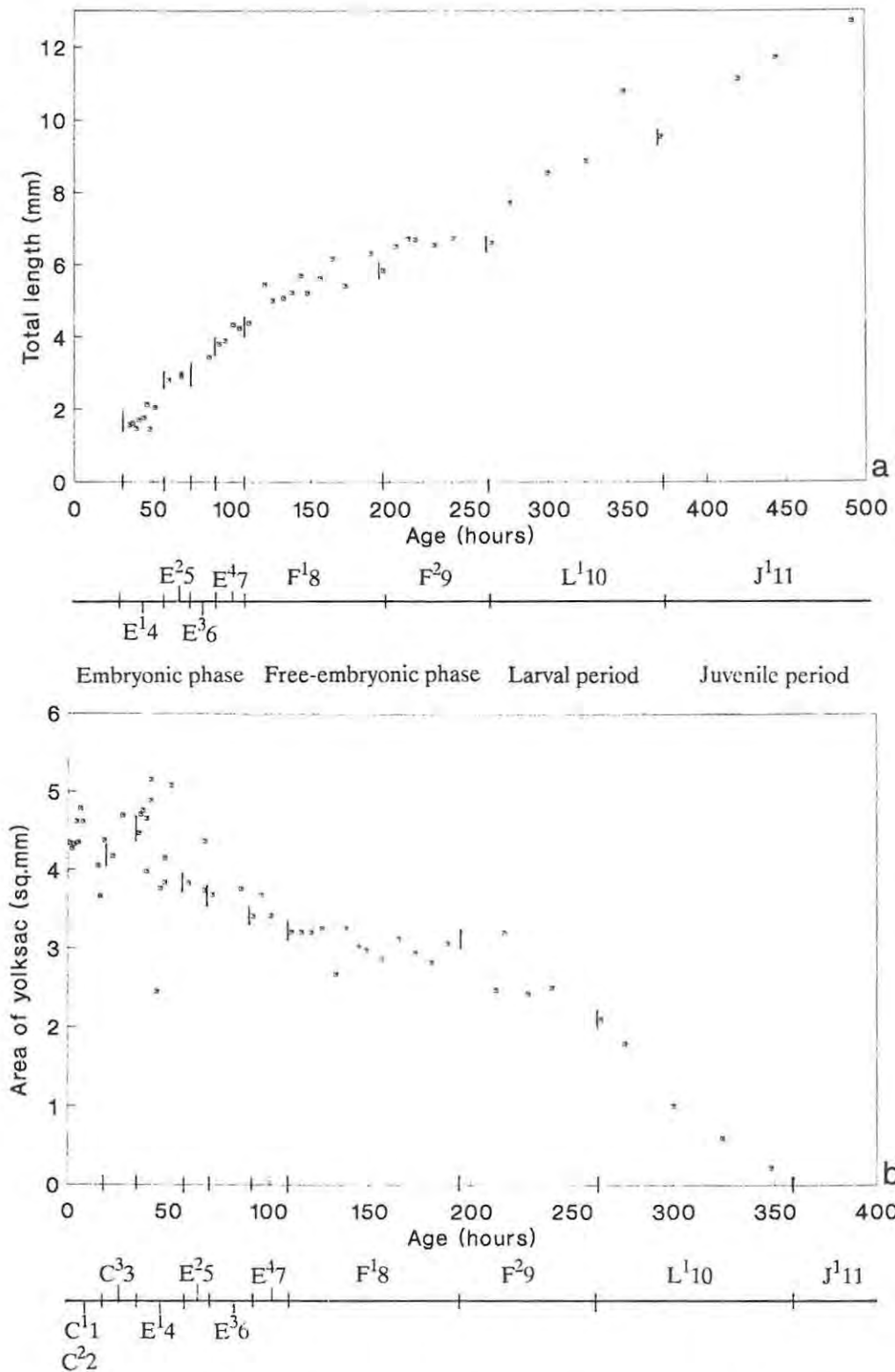
Figure 9 Growth in total length (a) and decrease in yolksac area (b) by steps and hours after activation of <u>Oreochromis</u> <u>mossambicus</u> embryos and larvae. The vertical lines represent step boundaries.
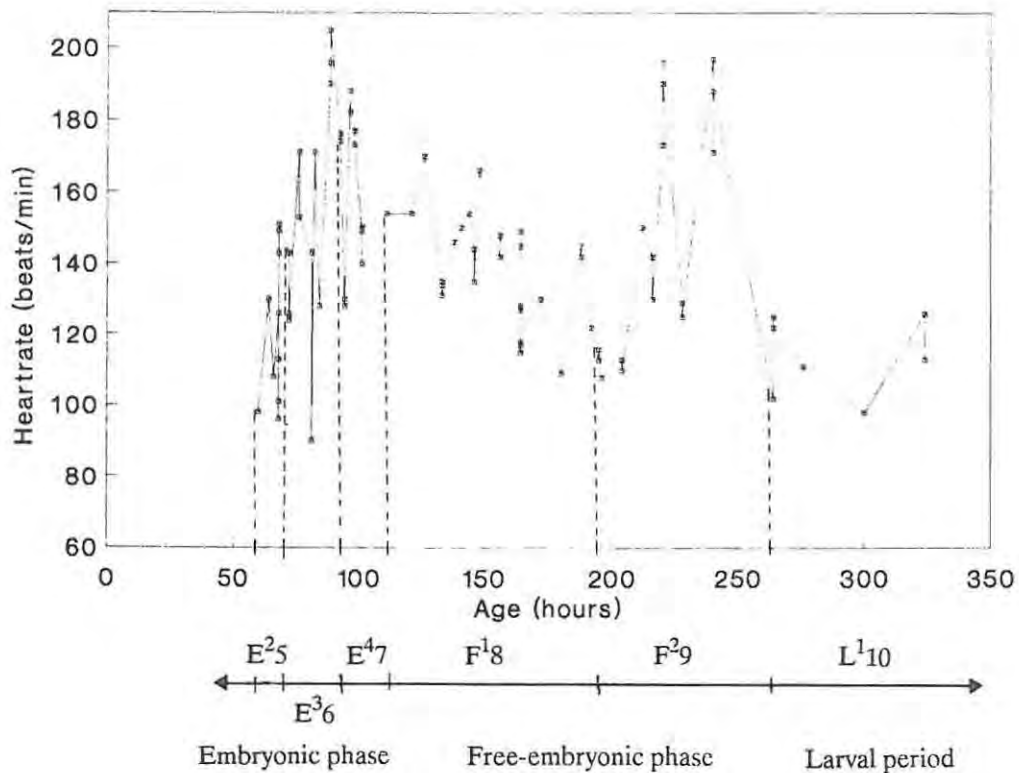
Figure 10 Heartrate by steps and hours after activation of _Oreochromis mossambicus_. The vertical bars represent step boundaries.

develop organs which subsequently have the potential to develop into organ-systems. In the later stages of the embryonic phase, the kinetic state is the obvious, visible activity of cardiac contractions, which occur at a step boundary. Release from the egg envelope could be interpreted as an increase in activity between the transition from the embryonic to the free embryonic phase.

Free-embryonic phase 04:16-11:00 (TU = 117 to 275)

This is an important transitional period in the early ontogeny of this species because it involves internal and external changes. Because most of the specimens had hatched at the

beginning of this step, it was considered to be the beginning of the free-embryonic phase. In dealing with mouthbrooders, the use of the term 'free-embryonic' could be misleading as it usually refers to newly hatched embryos/larvae which are free-swimming. In this instance, the embryos are free of the egg envelope but are not free-swimming as they are still incubated in the buccal cavity.

F^{1}8 04:16-08:04   The head became free from the yolksac. Intersegmental vessels and the caudal circulatory network were formed. The ventral finfold plexus reached maximum levels of development and declined by the end of this step as the anastomosing profundal caudal vein prepared to replace the inferior caudal vein. The heart chambers began to differentiate and take up their final position. Blood flow was first seen in the gill filaments and the pectoral fins. The vitelline plexus reached maximum development. First chondrification occurred in axial and appendicular skeletal components. Some dermal bone differentiation began. The mouth opened and jaw, pectoral fin and peristaltic movements were noted. The stomach, the spleen and the gall-, swim- and urinary bladders differentiated. Fin differentiation began. Iridocytes and retinal pigment were present.

Respiratory/circulatory systems: Because these two systems are so closely associated during early development, they shall be dealt with in one section.

At the beginning of this step anterior inter-segmental vessels had formed amongst the first eight pairs of somites (Figure 11a). The blood flowed dorsally from the region of the dorsal artery,

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looped above the notochord, and continued ventrally back towards the dorsal artery. The point of origin and destination was not visible. A small inverted U-shaped vessel lay between the eye and the gill pouches. A second caudal loop (the urostylar artery and vein) had formed ventral to the notochord and extended almost to its tip. This narrow vessel formed a large loop at the notochord tip, extended anteriorly, then joined the much thicker inferior caudal vein. At age 05:13 (TU = 139), a third caudal loop formed over the hypural area at a 90° angle to the notochord and the main urostylar branch. This marked the beginning of the caudal circulatory network. At this time, both the pre- and postanal finfold plexi reached a maximum degree of development and complexity (see Figure 11b). Figure 12 shows the development of the median finfold plexus from age 05:06 to age 06:00. The network of the postanal finfold resembled lacunae due to the profuse inundation of the vessels. In both finfolds, it was difficult to distinguish between veins and other tissues. Several hours later gill filaments were visible as semicircular pouches on the gill arches where blood was seen to flow dorsally (Figure 11b). Although the posterior intersegmental veins probably emptied into the postanal plexus at an earlier age, their pathway into the finfold network was clearly visible at this time.

By age 06:12 (TU = 163), blood flow was detected in some of the gill filaments (Figure 13a). The heart was situated posterior to the mouth and ventral to the branchial system. All four of the heart chambers were distinguishable within the pericardial cavity, as constrictions along the S-shaped heart-tube. Intersegmental vessels were present in all but the last few somites and the arteries formed loops dorsal to the spinal column. The caudal network had increased in complexity with up to four radial loops in the hypural region (Figure 13a). A large, open loop between the urostylar vessels and the ventral loops, was present in all of the specimens but the direction of the flow differed; sometimes the blood flowed dorsally and at other times it flowed ventrally. The level of complexity of the
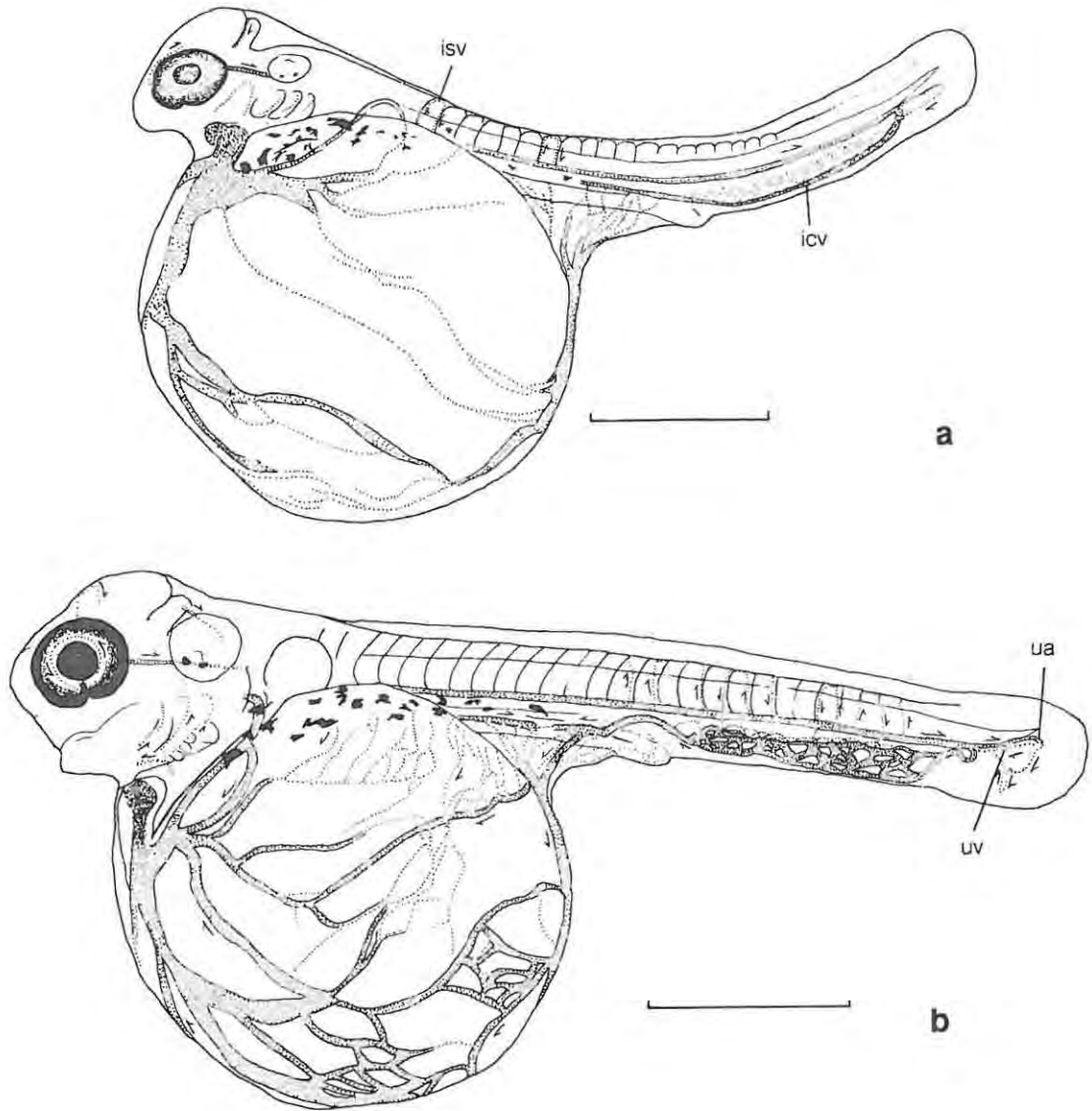
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Figure 11 Step $F^1 8$: a- age 04:16 (TU = 117), b - age 05:18 (TU
= 144) (icv = inferior caudal vein, isv = intersegmental vessel,
ua = urostylar artery, uv = urostylar vein). Scales = 1.0 mm.

caudal network was similar in all the specimens but the pattern and direction of flow was not. As the ventral finfold became more differentiated and the postanal plexus began to decline, the inferior caudal vein was positioned closer to the dorsal artery, especially in the anterior-most region. The inferior caudal vein continued flowing anteriorly beyond the preanal finfold and emptied into the anastomosing posterior cardinal vein. It lay ventral to the dorsal artery and extended anteriorly until it was no longer visible behind the tissue of the visceral cavity. The vitelline plexus had reached its maximum level of development and complexity, and completely covered all areas of the yolksac. The dorso-lateral surface of the yolksac consisted of a finer network of veins than that at the ventral aspect which contained a more gross network of larger vessels. The anterior vitelline vein had migrated forward and was visible along the anterior edge of the yolksac when viewed from the side. Although in most cases, the yolksac plexus was symmetrical, some individuals had a finer network on the left side. At age 06:20 (TU = 171) the subclavian vein was a single arched vessel in the pectoral fin. At age 07:12 (TU = 188) a marked reduction in the finfold plexus was noted. The replacement of the inferior caudal vein by the profundal caudal vein was underway. The profundal caudal vein was formed from the posterior segmental veins as they emptied into the postanal finfold plexus. The anastomosing profundal caudal vein lay ventral to the dorsal artery and extended anteriorly from the last somite to about mid-way along the postanal finfold. By age 07:20 (TU = 196) all that remained of the postanal plexus was the strongly flowing profundal caudal vein and a much reduced inferior caudal vein (Figure 13b & 14). The preanal plexus was also markedly reduced. Fine capillaries were present in the brain below the skin surface. Gill filaments were present on the first four ceratobranchials. Muscular development had begun in the ventricle which lay ventro-anteriorly to the atrium (Figure 13b). The segmental arteries and veins formed a two-tiered system, where loops and horizontal veins were formed dorsal to the notochord and at the junction of the dorsal finfold and the somites. Blood flowed through fine
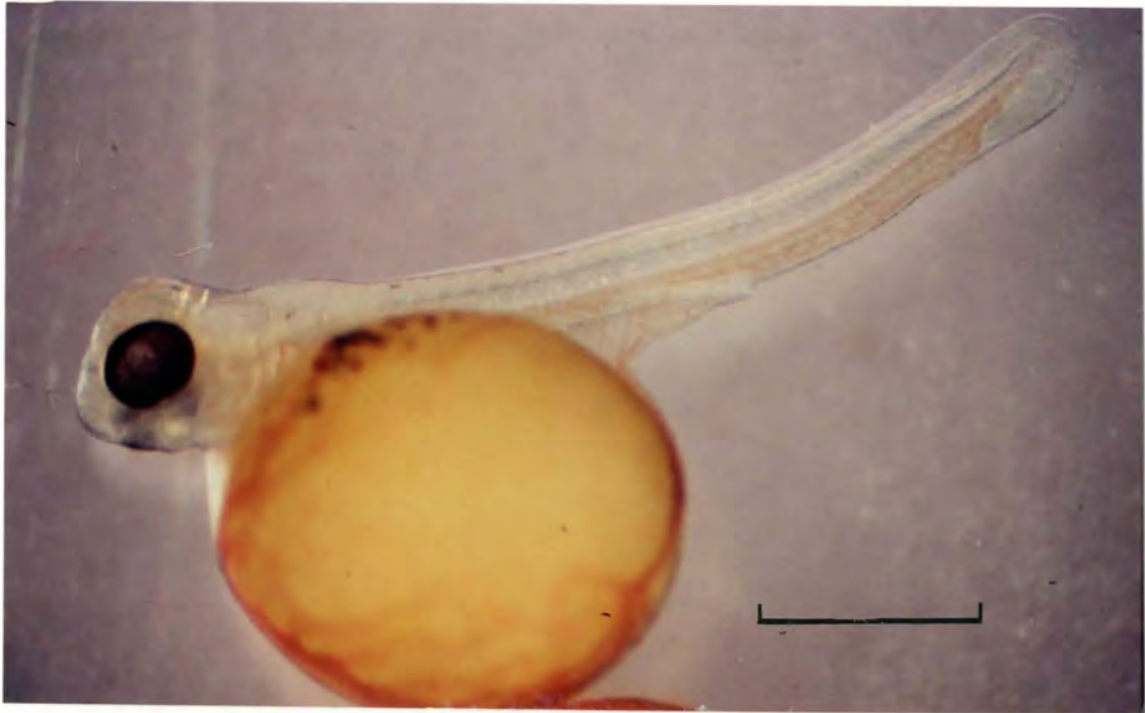
Figure 12 Step $F^1 8$: Top - age 05:06 (TU = 131), Bottom -age
06:00 (TU =150) showing the rapid increase in complexity of the
median finfold and vitelline plexi. Scales = 1.0 mm.
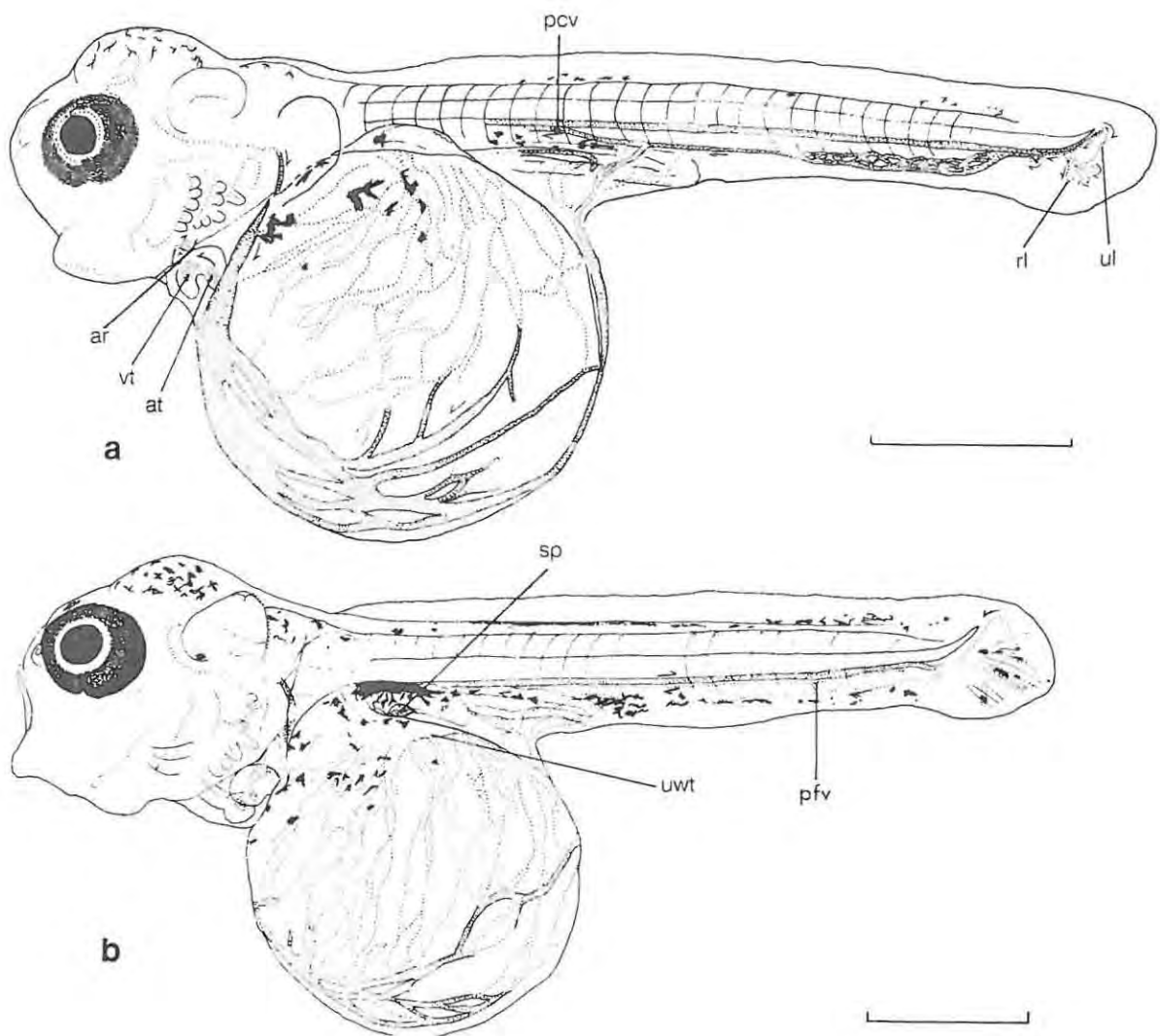
Figure 13   Step F¹8: a – 06:12 (TU = 163), b – 07:20 (TU = 196) (ar = arteriosus, at = atrium, pcv = postcardinal vein, pfv = profundal caudal vein, rl = radial loop, sp = spleen, ul = urostylar loop, vt = ventricle, uwt = unidentified white tissue). Scales = 1.0 mm.

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Figure 14 Step F¹8: An individual at age 08:00 (TU =200).
(The irregularly shaped object in the photograph is a fragment
of glass used to orient the embryo). Scale = 1.0 mm.

vessels in and around the developing gastro-intestinal tract.
Up to 10 radial loops had formed in the caudal fin and in one
instance there were two loops associated with one ray. The
subclavian vein had an additional arch.

Skeletal system: Mesenchymal aggregations in the ventral area
of the caudal finfold were noted at age 04:20 (TU = 121).
Cleared and stained specimens up to age 06:00 (TU = 150) retained
blue coloration in their tissue which made it difficult to see
skeletal components. The first indication that chondrification
had begun was at age 05:13 (TU = 139). Some alcian blue uptake
occurred around the ventral and lateral aspects of the developing
jawline and in the presumptive actinosal plates. The cleithrum
appeared as a thread-like, transparent structure. Five hours
later, alcian stain was noted in the ceratohyals, Meckel's
cartilage, in the region of the otic capsules and on the

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articulation process of the presumptive operculum. The angulo-articula and Meckel's cartilage appeared as one elongate structure which curved ventrally at its posterior end. Ventral to the slender palatoquadrate, the hyo-symplectic extended postero-ventrally to the otic capsule. The unfused trabeculae extended posteriorly and curved around the sides of the notochord. The parachordals were not clearly distinguishable but a transparent bar which curved dorso-anteriorly joined the presumptive basal plate to the floor of the otic capsule. Two ceratobranchials were visible as simple loops but had not retained any alcian stain. By age 06:04 (TU = 154) the ceratohyals articulated with the hyo-symplectic. The trabeculae flattened into the ethmoid plate anteriorly. Chondrification had begun in varying degrees in all the above four structures, as well as in the palatoquadrate and the angulo-articula (Figure 15a). The occipital arches extended dorsally from the parachordals. Further development of the branchial skeleton had occurred but individual skeletal structures were not distinguishable. Only the anterior margin of the otic capsules was stained. The presumptive maxilla was visible as a fine line in the tissue along the lateral jawline. The scapula-coracoid had a broad, triangular shape and the actinosal plate consisted mainly of a semicircular band; both were pale blue. The tissue around the outer margins of the pectoral fins and the caudal finfold was striated, indicating that actinotrichia would soon form.

By age 06:20 (TU = 171) two branchiostegal rays had formed and had retained some alcian stain. The ceratohyals broadened ventrally and differentiation of the interhyals had begun. The ventral hypohyals had begun to chondrify and were more advanced in their development than the dorsal ones. Chondrification had begun in all the ceratobranchials. Hypobranchials had not developed but the ventral point of the first three ceratobranchials, where they articulated with the basibranchial copulae, was broadened. The basibranchial copulae and cartilage were a single structure which extended posteriorly between the

ceratobranchials and was slightly stained in the anterior tip.
Two upper pharyngeal teeth per side were present. The trabeculae
were in contact with the parachordal plate but whether fusion had
occurred was not established. The posterior margin, and a bar
in the central regions of the otic capsule, were slightly
stained. The sclera formed a pale blue band around the eye. The
operculum had begun to fan out from the ventral point of its
articulation process. In the pectoral girdle, two to three
actinotrichia had begun to chondrify. The hypurals had begun to
differentiate into four distinctive structures; the dorsal
structure was barely perceptible, the middle one was crescent-
shaped and the ventral two were rectangular and stained darker.
Three actinotrichia had begun to form and chondrify adjacent to
the two middle hypurals.

At age 07:12 (TU = 188) the palatoquadrate curved proximally to
form a wing-shaped plate, with the dorso-anterior point lying
ventral to the eye ring and the trabeculae and the posterior wing
extending towards the hyomandibula (Figure 15 b-d). Dorsal to
Meckel's cartilage, the dentary had begun to form and contained
three minute teeth. The basihyal was faintly visible in the
branchial skeleton. The upper pharyngeal teeth consisted, on
each side, of a single anterior tooth and clumps of three to four
teeth of varying lengths, positioned posteriorly. All the teeth
were transparent and pointed in a slightly posterior direction.
The lower pharyngeal teeth were also in clusters of three to four
teeth of varying lengths on each side, but with anterior pointed
tips. The actinosal plate had begun to differentiate into a
dorsal and a ventral component. The lateral components
(arcualia) of 29 neural and 19 haemal arches had begun to develop
on the sides of the notochord. The opposed arcualia curved
towards each other but had not joined distally. Chondrification
had begun in all the arcualia but it was more predominant in the
posterior ones. Five hypurals were evident with the bases of the
first, second and third closely associated (Figure 15e). A
parhypural was faintly visible below the first hypural. Eight
caudal actinotrichia were forming. All of the above caudal
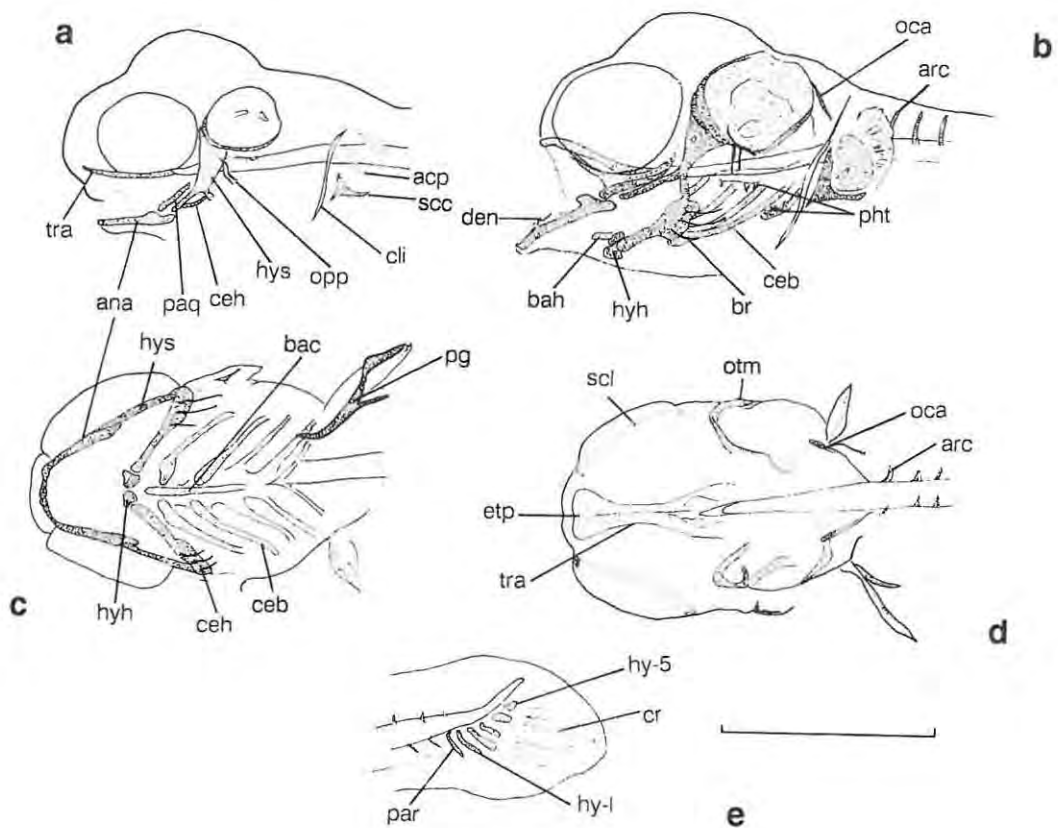
Figure 15: Step F¹8: Skeletal development and chondrification. a - left lateral view of the head at age 06:04 (TU =154); b - left lateral, c - ventral and d - dorsal views of the head at age 07:12 (TU = 188); e - caudal fin at age 07:12. The degree of alcian blue uptake in the individual structures is represented by the stippled areas (acp = actinosal plate, ana = angulo-articula, arc = arcualia, bac = basibranchial copulae, bah = basihyal, br = branchiostegal rays, ceb = ceratobranchial, ceh = ceratohyal, cli = cleithrum, cr = caudal ray, den = dentary, etp = ethmoid plate, hyh = hypohyal, hys = hyo-symplectic, hy-1 = hypural 1, hy-5 = hypural 5, oca = occipital arch, opp = opercular process, otm = otic capsule margin, paq = palatoquadrate, par = parhypural, pg = pectoral girdle, pht = pharyngeal teeth, scc = scapula-coracoid, scl = sclera, tra = trabeculae). Scale = 1.0 mm.

structures were stained blue to some degree.

Other systems: By 04:20 (TU = 121) the head was free of the yolksac. The mesencephalon and the cerebellum extended dorsally giving the head a more rounded configuration. The mesencephalon began folding posteriorly and lobular development had commenced. Further differentiation in the rhombencephalon made the cerebellum and the medulla oblongata easily discernible. The division of the prosencephalon into the diencephalon and the telencephalon had occurred and the nasal channels had opened. The curvature of the body axis had decreased, resulting in a straighter head and trunk alignment but the tail remained dorsally curved. Internal development had begun in the otic capsules. The anterior margin of the otic capsules touched the ventro-posterior margin of the cerebellum, therefore reaching their final position.   Some iridocytes were present in the eyes.   A narrow line along the presumptive jawline at age 05:01 (TU = 126) marked the external opening of the buccal cavity and the initial formation of the mouth. The eyes were black and retinal pigmentation was pale at age 05:13 (TU = 139).   Up to this time body pigmentation was confined to a few melanophores along the body axis dorsal to the preanal finfold and along the dorsal margins of the somites. The body axis was linear and the yolksac was markedly reduced. At age 05:18 (TU = 144) the eyes were golden and the retinas were darkly pigmented (see Figure 12).   Between ages 06:00 (TU = 150) and 07:00 (TU = 175) stellate-like melanophores had developed above the forebrain, the midbrain and the medulla oblongata.   A few iridocytes were present over the swimbladder, the visceral cavity and along the lateral flanks.   The mouth had formed, jaw and pectoral fin movement was noted and the urinary bladder became visible.   The presence of a yellow-green structure, more clearly visible on the right side of the embryo, indicated that the gall bladder had formed. The pectoral fins had rotated and lay perpendicular to the body axis and posterior to the operculum which covered the third gill arch.   Only one individual during this step had a yellow substance present in the gastro-intestinal tract; this

occurred at 07:20 (TU = 196). The oesophagus was a thick-walled, muscular tube with two distinctive regions. The remainder of the digestive tract consisted of a thin-walled tube which narrowed posteriorly; the stomach and hindgut were separated by a slight constriction. The convolutions of the gastro-intestinal tract went from left to right and ascended slightly to run ventral to the notochord. In cleared specimens a kidney-shaped organ emptied via a duct into the foregut, confirming that the gall bladder had formed. The spleen had formed and an unidentified thick white tissue lay along the rim of the yolksac and the visceral cavity (Figure 13b). A fine, transparent thread-like exudate extended out of the anus of one specimen at age 08:00 (TU = 200) and three others at age 08:03 (TU = 203) which indicated that the gut lumen was open at the anus. Peristaltic movements had begun. The swimbladder was visible above the visceral cavity as a small, ovoid chamber with thick walls and a slit-like lumen. The ventral finfold was reduced and differentiation had begun in the caudal finfold (see Figure 14). Pigmentation extended along the dorsal and ventral body line and a few melanophores had formed on the roof of the pericardium. The pigments on the dorsal surface of the head had begun to aggregate. The tissue covering the visceral cavity was heavily pigmented with brown, green and yellow melanophores as well as iridocytes. The eyes were profuse with iridocytes and a few were present in the opercular area. A few melanophores were present along the presumptive caudal fin rays (Figure 13b & 14). Throughout this step the dorsal finfold increased and reached a maximum size by the end of this step.

As in all the previous steps, heartrate fluctuated without any apparent pattern. However, there was a general trend towards a decrease in the heartrates (Figure 10). The largest increase in embryo length (1.9 mm) of any previous step was recorded. There was no change in yolksac area (Figure 9).

Step summary: The main ontogenetic events occurred in the circulatory, respiratory and digestive systems. The basic

skeletal components were laid down. The major site of gaseous exchange up to this point, the ventral finfold plexus, reached maximum development as the permanent adult respiratory structures increased in complexity but were still incomplete. Concurrently, the second embryonic respiratory network, the vitelline plexus, also attained maximum complexity but lagged behind that of the ventral finfold plexus. Muscular development in the ventricle ensured stronger flow into the rapidly developing vessels in the gastro-intestinal tract, the myotomes, the pectoral fins and the caudal, finfold and vitelline networks. Skeletal development had been predominantly in the suspensorium, the hyoid arches, the pectoral girdle and the caudal fin skeleton. There was an overall increase in body pigmentation and coloration. Functional changes were evident in pectoral, jaw and peristaltic movements, and the presence of the yellow substance in the gut lumen.

$F^2$9   08:04-11:00   The inferior caudal vein was replaced by the profundal caudal vein and the finfold network disappeared. There was a marked decline in the yolksac plexus and the secondary gill lamellae were formed and vascularized. There was increased dermal bone differentiation. The neural and haemal arches fused at their distal ends, and caudal fin lepidotrichia and proximal pterygiophores in the median fins were formed.

The inferior caudal vein was no longer present at the beginning of this step. The profundal caudal vein emptied directly into the posterior cardinal vein which continued anteriorly until it was no longer visible behind the yolksac and the tissue of the visceral cavity. The preanal plexus consisted of a few vessels supplied with blood from a vein leading from the junction point of the profundal caudal vein and the posterior cardinal vein. These preanal finfold veins fed into one large vein which flowed anteriorly and supplied blood to the digestive system. The

veins of the vitelline plexus formed a symmetrical pattern over the entire surface of the yolksac (see Figure 16). The veins leaving the body were of a uniform thickness and emptied into one major collecting vessel along the ventrum of the yolksac. The anterior vitelline vein was no longer present and it appeared that the common cardinal vein entered the ducti Cuvieri directly. The second and third ceratobranchials had double gill filaments. By age 08:12 (TU = 213), secondary lamellae began to form on the gill filaments of the second and third ceratobranchials. Twelve hours later, blood vessels had formed in the secondary gill lamellae. The tissue above the anterior half of the visceral cavity was covered in iridocytes and yellow pigments, whereas the posterior half was transparent. The preanal finfold had disappeared. The anal, dorsal and caudal fins began to take shape. The gastro-intestinal tract was strongly convoluted (Figure 16).

At age 09:04 (TU = 229), four siblings were sampled which showed a wide range of variation in their level of development. When such differences occur, the descriptions will be of the individual most closely following the modal level of development and only a brief reference will be made to the variation. Further discussion of variation amongst similar aged individuals will follow later.

By age 10:04 (TU = 254), iridocytes extended posteriorly over the visceral cavity to the anus and ventro-laterally over the yolksac. A few pigment cells were present along the notochord and there was an increase in melanophores above the medulla oblongata and the first few somites. The heart had reached its final position. Reduction of the yolksac gave it the appearance of a sphere extruding from the stomach of the embryo. Differentiation in the median finfolds had begun posteriorly, making the caudal finfold markedly distinctive. The development of the digestive system made it possible to distinguish between the oesophagus, the stomach, the intestines, the spleen and the gall bladder. Pouch-like projections were visible along the
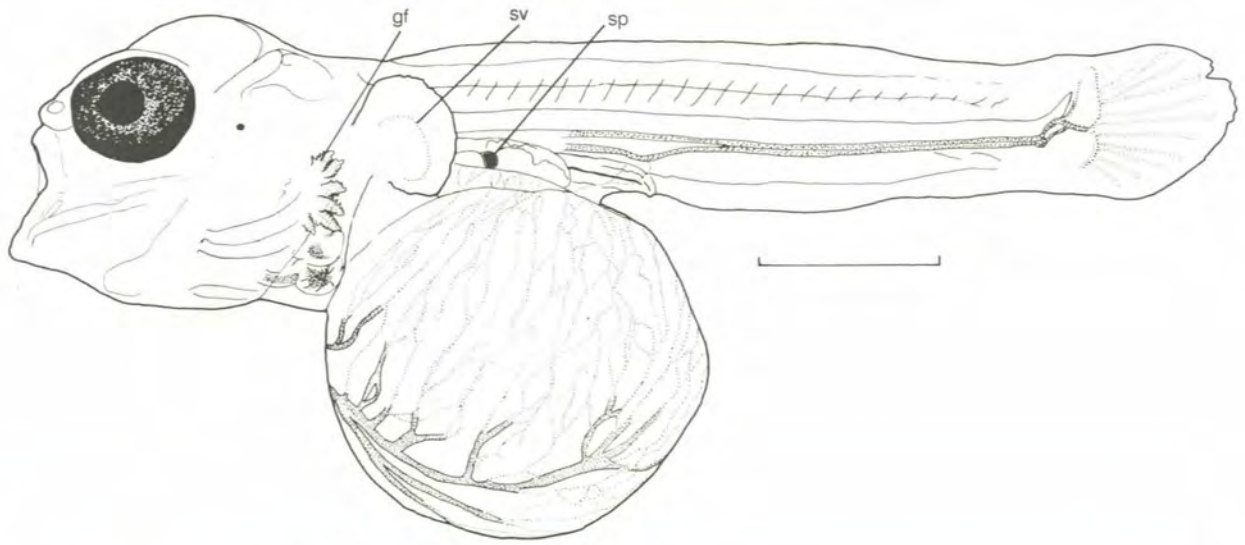
Figure 16 Step F²9: Top - at age 09:00 (TU = 225); b - at age 10:00 (TU = 250) (gf = gill filament, sp = spleen, sv = subclavian vein). Scales = 1.0 mm.

lining of the stomach and the intestine.

As with the last few specimens of the former step, there was a transparent thread-like exudate extending out of the anus in the first specimen of this step. This exudate was not noted at any other time. As the function and origin of this exudate was not determined, its presence was not considered to be an indication of exogenous feeding. Possibly it was the sloughing off of mucous, or some cellular material, from the walls of the gastro-intestinal tract. There was no food visible in the digestive tract of live specimens during this step. Examination of cleared/stained specimens revealed the presence of an unidentified ovoid-shaped object, thought to be some kind of planktonic crustacean, in the digestive tract of embryos from age 08:17 (TU = 204) onwards. Initially there was only one zooplankton present but with time a gradual increase in numbers was noted. From age 09:00 (TU = 225), a granular material was present in the digestive tract. At age 10:04 a round red object resembling an otolith and a blue structure were present in the gut. This may have been an indication of cannibalism. The abundance of these planktonic animals was not sufficient to contribute to the nutritive requirements of the embryos.

Embryos in the incubator tank were relatively inactive and responded passively to the inflow at the mouth of the funnel where most of them were gently tossed around. Initially embryos that were placed in the incubator cups were also inactive and lay passively in clusters on the bottom of the cup. By age 10:23 (TU = 274), several individuals swam inefficiently around the bottom of the cup or momentarily in the water column, but the majority were still clustered at the bottom. When food was added to the funnels and the cups, the embryos did not appear to be feeding.

Throughout this step, peristalsis was noted and yellow substance production increased. Initially the yellow substance was present only in the foregut, but with time it was also noted in the

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hindgut and eventually several specimens expelled the yellow substance through the anus while under observation. All these events were indicative that the digestive system was approaching a functional state.

Skeletal system: At the beginning of this step, the hypobranchials had begun to differentiate and the gill filament rays had begun to chondrify. Gill rakers were visible. The basihyal was present and flattened anteriorly. The ceratohyals were spatulate dorsally. Four branchiostegal rays were present. The dentary had taken up some alcian stain and extended posteriorly towards the maxilla. The angulo-articular had begun developing processes at the articulation point with the palatoquadrate. A perforate structure with ragged edges lay between the dentary and Meckel's cartilage. The premaxilla was visible as a transparent, ragged, fine line. The maxilla curved posteriorly at the dorsal end. The dorsal outline of the suboperculum had stained blue. The margin and the outer walls of the otic capsules, as well as some of its internal structures, had begun to chondrify. The blue-stained occipital arches extended anteriorly towards the otic capsules. Various processes extended dorsally from the neurocranial floor and connected with the otic capsules. The parachordals extended anteriorly between the trabeculae but were not stained. The ethmoid cartilage extended dorsally towards the paraphyseal bar. The epiphyseal bar and the anterior and posterior orbital bars had all taken up some alcian stain. A foramen had developed in the upper portion of the scapula–coracoid and the posterior region had begun to elongate.

At age 08:17 (TU = 218), the tips of all of the pharyngeal teeth had taken up alizarin stain. The upper pharyngeal teeth consisted of two anterior teeth and a cluster of 10 to 12 posterior teeth per side. The pharyngeal bones (the fused fifth ceratobranchials) contained clusters of 10 to 12 teeth per side. The parachordals extended posteriorly where they enclosed the notochord ventrally and laterally. When viewed laterally the

floor of the otic capsule was seen to dip ventrally, forming the chamber for the sacculus. This was indicative of basal plate formation. The supracleithrum appeared as a transparent, thread-like bone extending anteriorly at an angle of 45° from the cleithrum. Fusion of the right and left extensions of the neural and haemal arches had begun in the posterior, while the anterior arches remained separate. Hypurals one to three were fused at their bases. There were six actinotrichia and six double-segmented lepidotrichia present in the caudal finfold.

At age 09:00 (TU = 225) alizarin stain was retained in the branchiostegal rays, the dentary, the tips of the anterior dentary teeth, the maxilla, and the operculum process. The saggita otolith had a pink tinge. This was the first time that any otoliths had been observed in the cleared and stained specimens. They were known to be present since step E^25 at the age of 02:20 from observations of live specimens. The apparent absence of otoliths in the cleared samples could be attributed to the preservation solution, despite the precautions taken to avoid decalcification by using a buffered formalin solution. Over the next 24 hours some degree of variation in the level of calcification was noted in the samples taken.

At age 09:12 (TU = 238) two teeth had formed on the premaxilla, the ascending premaxillary process extended dorso-posteriorly towards the paraphyseal bar, four radials had begun to form from the actinosal plate. There were three mesenchymal rays in the pectoral fins and the tissue of the median finfolds was striated due to the density of aggregating mesenchymal cells. By age 09:20 (TU = 246), most of the neural arches had fused and over fifty percent had developed spines. All except the first few anterior haemal arches were fused. Three hours later, proximal pterygiophores had begun to form and chondrify along the base of the dorsal finfold; the middle ones were more advanced in their development. Figure 17 illustrates the overall skeletal development at age 09:20 and 10:04.
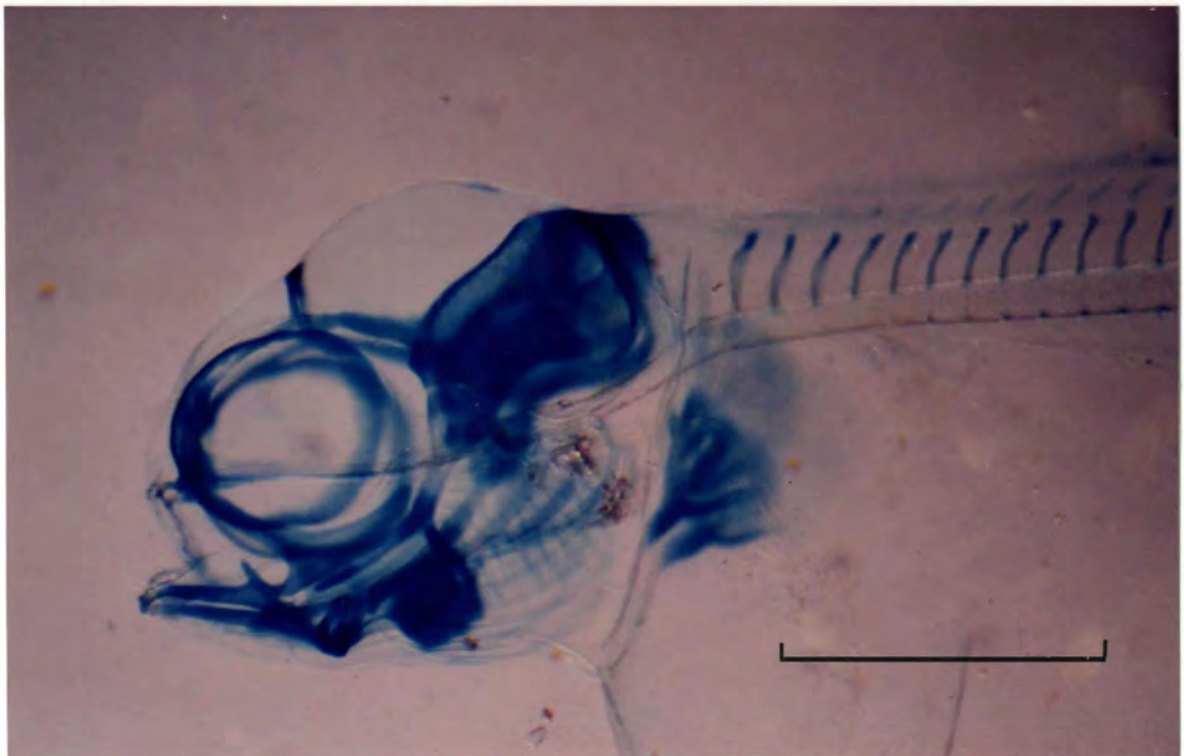
Figure 17 Step $F^2$9: Top - at age 09:20 (TU = 246), Bottom - at age 10:04 (TU = 254) showing skeletal development. Calcification in the structures does not show up. Scales = 1.0 mm.

By age 10:04 (TU = 254), elements of the branchial skeleton had become more distinguishable due to increased development and chondrification (Figure 17). The epibranchials and the three hypobranchials had all differentiated. The basibranchial cartilage consisted of two separate components. The first section extended posteriorly from the hypohyals to the third hypobranchial and the second component lay between the fourth ceratobranchial and the lower pharyngeal bone. A notable increase in calcification had occurred. The dentary had begun to encircle and calcify around Meckel's cartilage. Six dentary and four premaxillary teeth were calcified. The maxilla flattened dorsally and both it and the premaxilla had taken up some alizarin stain. The hyo-symplectic was constricting at its articulation point with the interhyal and a furrow had formed in the dorsal region where the two individual bones of this complex would differentiate. The outer walls of the otic chamber were blue and five inner compartments were visible. Differentiation had begun in the operculum and the suboperculum. Calcification had begun along the margins of the parasphenoid, around the anterior tip of the notochord, and along the occipital arch. A septum had formed below the epiphyseal bar. The paraphyseal bar was thickened at its junction with the anterior orbital bar which extended ventrally to join up with the ethmoid cartilage. The posttemporal lay between the supracleithrum and the posterior wall of the neurocranium, and the cleithrum had begun calcifying. Three ribs were visible as fine, blue lines. The notochord was slightly pink in the regions of the haemal arches with an increased intensity at the points of contact between the arches and the notochord. The urostyle had begun calcifying. One epural was present posterior to the last neural arch. Calcification in the 16 caudal rays indicated that lepidotrichia were forming. In one specimen, there were up to four segments in the caudal fin rays. Five proximal pterygiophores had begun to form in the anal finfold and dense mesenchymal aggregations, precursors of actinotrichia, were present in the center of both median finfolds.

The heartrate was erratic but peaked at the end of the step (Figure 10). There was no growth and the yolksac area remained constant (Figure 9).

The regression analysis on yolk area versus time and embryo length versus time suggested that at least two regression regimes exist (Figure 18). A significant F ratio indicated that the slopes and intercepts differ beyond chance between the groups ($F = 7.5$, $p = 0.00298$ for embryo length and $F = 19.4$, $p = 0.00003$ for yolk area). The point estimate of the breakpoint, was that point separating the two regimes which gave the most significant values of the F ratios for testing for equality of regressions. The change point thus derived was at the sixth point from the end of the two sequences. This indicated that the actual breakpoint is somewhere between the sixth and eighth points. These points occur at ages 09:00, 09:12 and 10:00. Although it cannot be stated at what age the exact breakpoint occurs, it is more likely to be at age 09:12. This breakpoint coincides with an interval of no growth, no change in yolksac area, a peak in heartrate and the transitional state between endogenous and mixed feeding (Figures 19 & 20).

Step and phase summary: Marked changes have occurred in the digestive, respiratory and circulatory systems, all of which appear to be close to completion. Vascularization, differentiation and chondrification of the branchial apparatus and the disappearance of the embryonic finfold plexus marked the switch to adult, branchial respiration. Gaseous exchange was still supplemented by the reduced vitelline plexus. The large yolksac served as a supplementary food source. All the adult skeletal structures were present. Differentiation and chondrification was most pronounced in structures associated with feeding and locomotion.

The peak in heartrate and the lack of increased embryonic length during the breakpoint interval can be attributed to increased morphological complexity, oxygen demand, metabolic processes and

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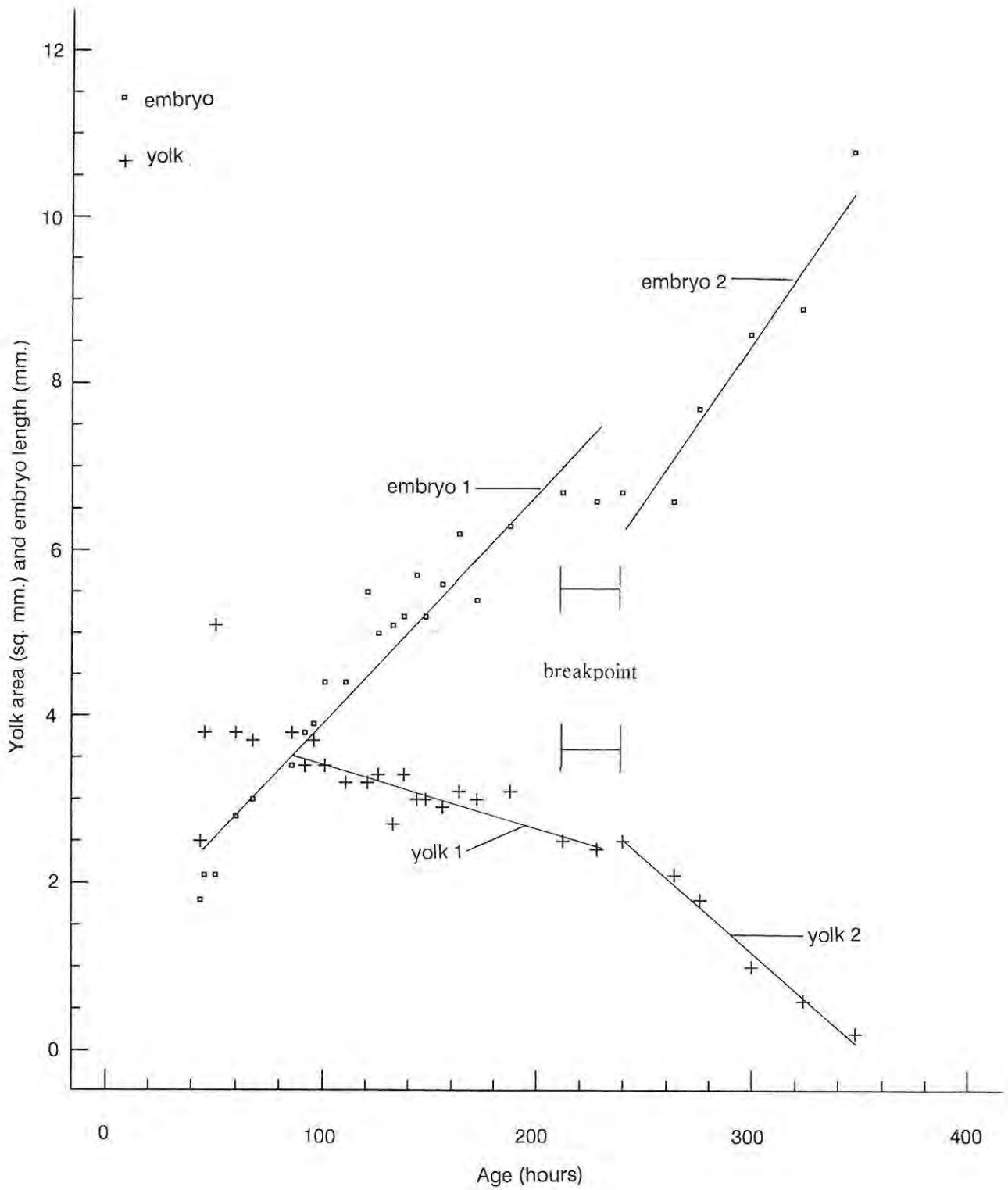
Figure 18 Regression analysis of yolk area versus time and embryo length versus time showing the breakpoint which occurs between ages 216 hours (09:00) and 240 h (10:00). The two regimes for both the variables are labelled as embryo 1 & 2, and yolk 1 & 2.
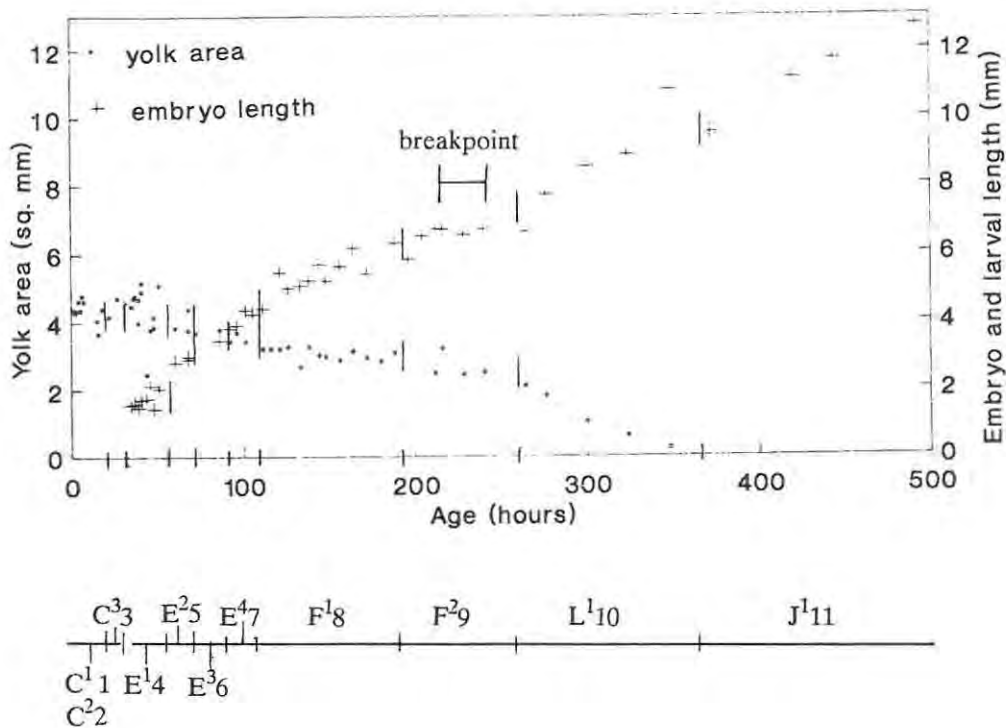
Figure 19 Embryo length and yolk area changes with time. The breakpoint occurs at a crucial point of transition between ages 216 hours (09:00) and 240 hours (10:00). The vertical bars represent step boundaries.

their by-products, and a subsequent decrease in oxygen availability. Energy is being used for structural development rather than for growth. More oxygen is required for metabolic processes as the complexity of the embryonic structures increases. Subsequently metabolic and respiratory by-products increase and their excretion into the buccal cavity may decrease the available oxygen. The metabolic rate and cardiac contractions increase to compensate for declining oxygen levels and increasing oxygen demands. This cyclical situation continues while the embryos remain in the buccal cavity.
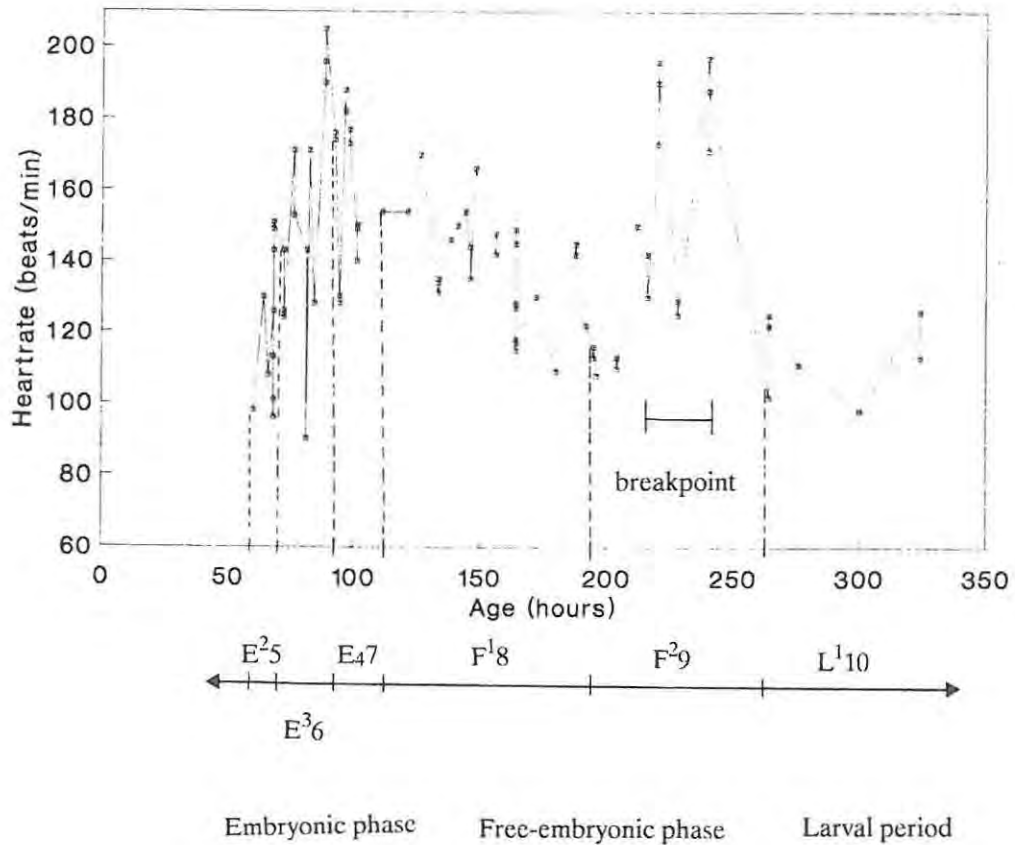
Figure 20: Heartrate by step and hours after activation showing the ages at which the breakpoint occurs (between ages 216 hours = 09:00 and 240 hours = 10:00)

Within the free-embryonic phase, the step boundaries are not obvious. The boundary between the embryonic period and the larval period is distinct but the exact timing is masked by variation. There is evidence that the threshold between the these periods is saltatory in nature. Morphologically,

functionally and behaviourally the embryos are prepared for the marked change about to occur in their external environment and life-history style. This change places the young in a highly vulnerable position. The breakpoint data clearly indicates a rapid, leap from one level to another (Figures 18-20).

The complexity of the embryos makes the analogy of potential and kinetic activity difficult to assess. The morphological development of the organ systems, especially those of digestion, respiration and circulation, can be equated with activity, and the point of functionality as the potential. This potential is then expressed in the larval period when these systems begin functioning.

Larval period 11:00-15:00 (TU = 275 to 375)

Mixed feeding, endogenously from the yolksac and exogenously through ingestion, marks probably the most important transitional period in the early ontogeny of O. mossambicus. The larval period encompasses this transition. The switch from endogenous to exogenous nutrition was completed at about age 15:00 (TU = 375), thus marking the end of the larval period and the beginning of the juvenile period.

$L^1$10 11:00-15:00   First exogenous feeding occurred at the onset of this step. Ossification of cartilage bone, median, caudal and pectoral fin lepidotrichia and vertebrae began. The swimbladder filled and pelvic fin buds were formed. Enclosure of the yolksac and finfold differentiation was almost completed. Neuromasts and cupola formed along the caudal peduncle. Melanophores were present in all the fins.

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The main developmental descriptions during this period centered around the skeletal system, as most of the other organ systems were either not visible, and/or were relatively complete in their development. The foundations of all the organ systems had been laid down and some level of functionality had been attained.

Within the first hour of this step differences in the level of development of four siblings were noted. The smallest individual was less developed than specimens 26 hours younger. Further discussion on this variation follows later.

The elimination of a dark, particulated faeces and the presence of large quantities of zooplankton in the intestinal tract of the specimen aged 11:00 (TU = 275) were the first positive indication that exogenous feeding had begun. The yolksac was spherical and large relative to body length, and the vitelline plexus formed a uniform network over the entire yolksac surface (Figure 21a, see also Figure 22). At age 12:12 (TU = 313) the yolksac was still relatively large and bulged below the ventral body line (Figure 21b), but by 13:12 (TU = 338) a marked reduction of both the yolksac and the embryonic finfold was noted. By age 14:12 (TU = 363) the yolksac was visible as a slight extension ventral to the body line and the vitelline plexus consisted of a few vessels, a pinkish area, or simply as channels without any flow (Figures 21c & 22). In most specimens all that remained of the yolksac could be seen through a narrow gap between the opposing sides of the descending body wall tissue (Figure 23a). The yolk that was present in the visceral cavity was a remnant of the yolksac and probably contributed very little, if at all, to the nutritional requirements of the larvae. This marked the end of endogenous feeding and, consequently, was the termination of mixed nutrition.

At the beginning of this step larvae had begun to swim in the water column of the funnel. Within 27 hours a large proportion of individuals were free-swimming in the upper portions and upwards along the walls of the funnel (possibly feeding), and

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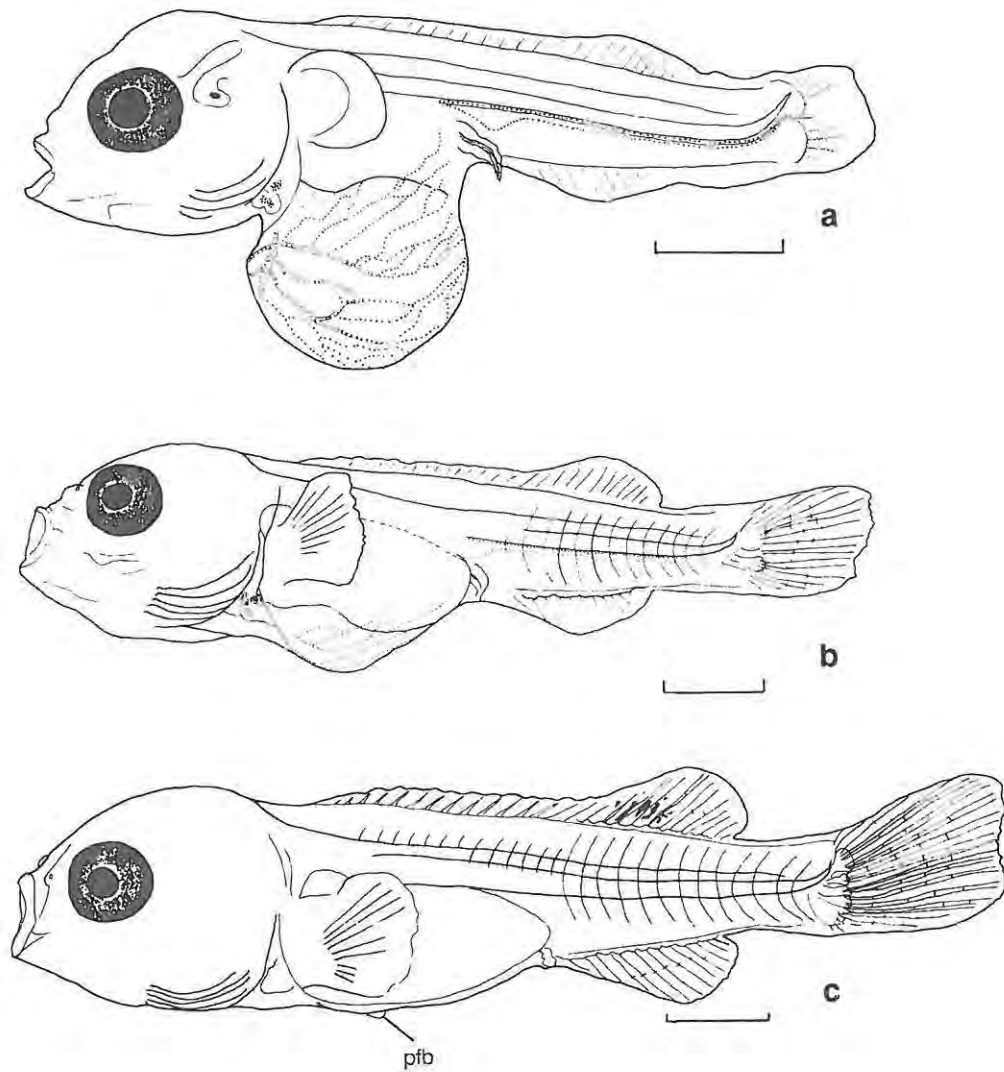
Figure 21 Step L¹10: a - at age 11:00 (TU = 275), b - at age
12:12 (TU = 313), c - at age 14:12 (TU = 363). Note the rapid
decrease in the yolksac as the body wall descends and the
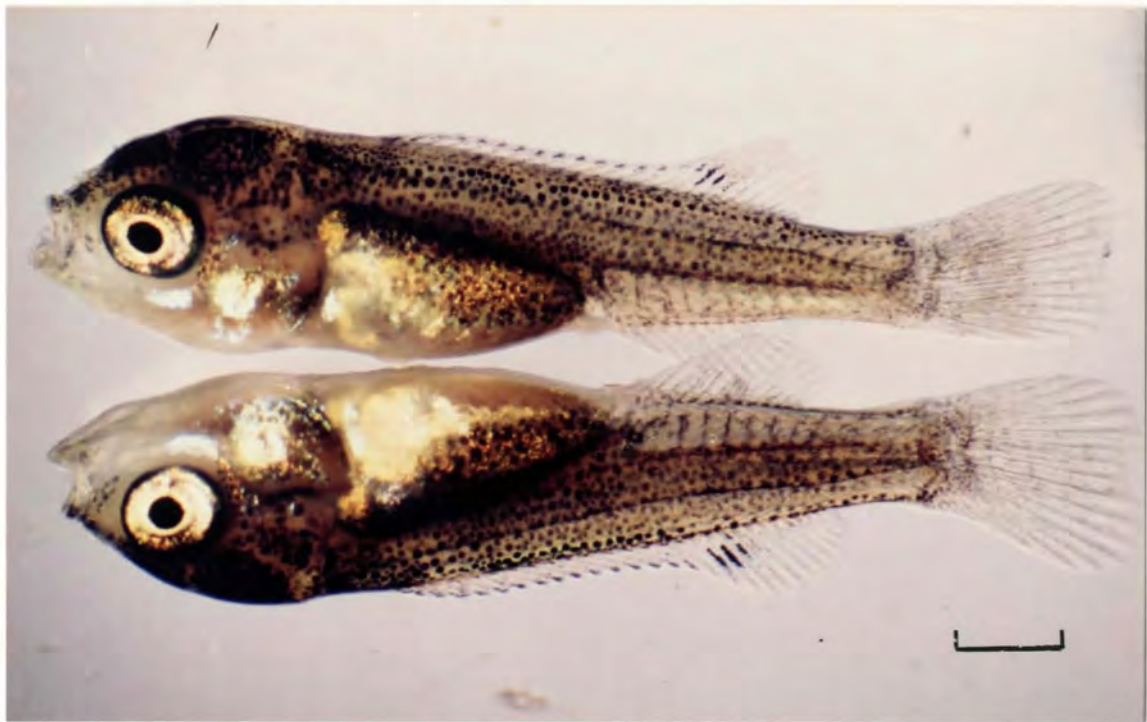differentiation of the fins (pfb = pelvic fin bud). Scales = 1.0
mm.

Figure 22 Step L¹10: Top - at age 11:12 (TU = 288) and bottom at age 14:12 (TU = 363) showing rapid yolksac enclosure and pigmentation development during the truncated larval period. Scales = 1.0 mm.

some had made their way through the outflow tube into the cups. By age 12:12 (TU = 313) the larvae appeared to be feeding and by age 13:12 (TU = 338) they were actively pursuing brine shrimps in both the funnels and the cups. By that time most of the individuals had swam into the cups.

At the beginning of this step fine, thread-like melanophores extended laterally along the flanks of the body, and the dorsal regions of the head were heavily pigmented. A few pigment cells were present along the posterior base of the dorsal finfold (see Figure 22). The walls of the swimbladder were thinner and the lumen had begun filling with air.

Dechondrification in the central regions and calcification along the margins of the hypurals, the interhyals, the ceratohyals, the hyo-symplectic and the palatoquadrate were evident (Figure 24). The third and fourth pharnygobranchials had begun to calcify. There were three teeth on the second pharyngobranchial and the posterior clumped teeth were arranged in rows on the third and fourth pharyngobranchial. Other structures which had begun calcifying were the pharyngeal bone, the midline of the urohyal, the lepidotrichia of the pectoral fins, the bases of the neural and haemal arches, the urostyle and the anterior tip of the notochord. Faint pink banding on the notochord indicated that vertebral development had commenced. The maxilla had fused with the premaxilla. A cartilaginous bar extended antero-ventrally beyond the junction of the paraphyseal bar and the anterior orbital commissure, where it almost touched the anterior tip of the palatine. Development of the parietal had begun as evidenced by two opposing bones forming along the dorsal roof of the neurocranium; one bone extending posteriorly from the epiphyseal bar and another bone extending anteriorly from the dorso-posterior wall of the neurocranium. The preoperculum had begun forming and lateral line pores were present along its dorsal rim. Posterior constrictions of the median finfold made the shape of the differentiating dorsal and anal fin obvious. Mesenchymal rays had formed in the median fins and aggregations
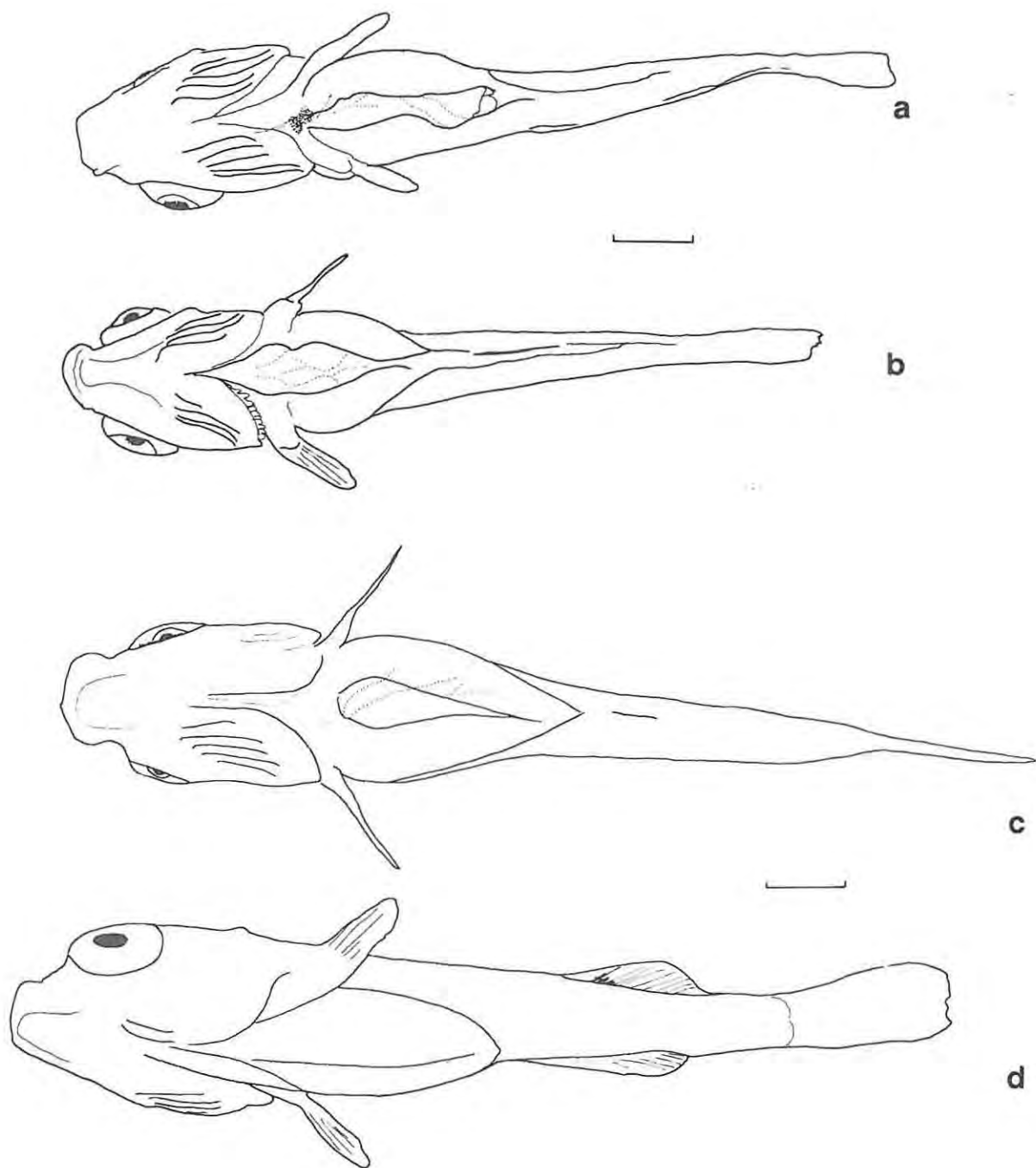
Figure 23 Ventral views showing migration of the body wall over the yolksac; a - at age 14:12 (TU =363), b - at age 15:12 (TU =388), c - at age 15:00 (TU = 375), d - at age 16:18 (TU = 422). Scales = 1.0 mm.

of mesenchyme along the leading edges of the descending body wall were the first signs of pelvic fin formation. Figure 24 and 25 illustrate the skeletal development at age 11:01.

Over the next three and one half days increased development and calcification was noted in the oro-nasal area. The vomer was calcified and had a ragged appearance along the ventro-anterior surface. Eight ribs were present. All three otoliths were visible in the otic capsule. The vertebrae at the extremities of the notochord were distinguishable and had begun to calcify. The postcleithrums and one uroneural had formed and began calcifying. Two epurals were present. Distal pterygiophores and up to 28 rays, some with two segments, had formed in the dorsal fin, and in the anal fin there were up to 12 lepidotrichia, some with two segments. A cleared and stained specimen revealed that there were 3 spines and 10 rays present in the anal fin. The proximal pterygiophores extended farther into the body tissue and were positioned between the haemal and neural arches. Calcification had begun in the median fin rays. The caudal fin had up to 22 lepidotrichia with up to five segments. The pectoral fin had 12 rays with up to three segments. All the fin rays extended to the distal margins of their respective fins. The pelvic girdle was an undifferentiated fine line with flaps of striated tissue extending from it. There was a concentration of pigments along three of the dorsal fin rays which denoted the beginnings of the characteristic 'tilapia spot'. A few melanophores were present along some of the anal and pectoral fin rays, along the distal membrane of the dorsal fin, and throughout the caudal fin. The entire body was covered in pale, stellate melanophores (Figure 22). Some green and yellow coloration, as well as iridocytes, were also noted. At least twelve neuromasts and cupola were present along each side of the caudal peduncle. At age 14:12 (TU = 363) white rings separated the developing vertebrae which were slightly concave.

There was a marked drop in the heartrate (Figures 10 & 20).
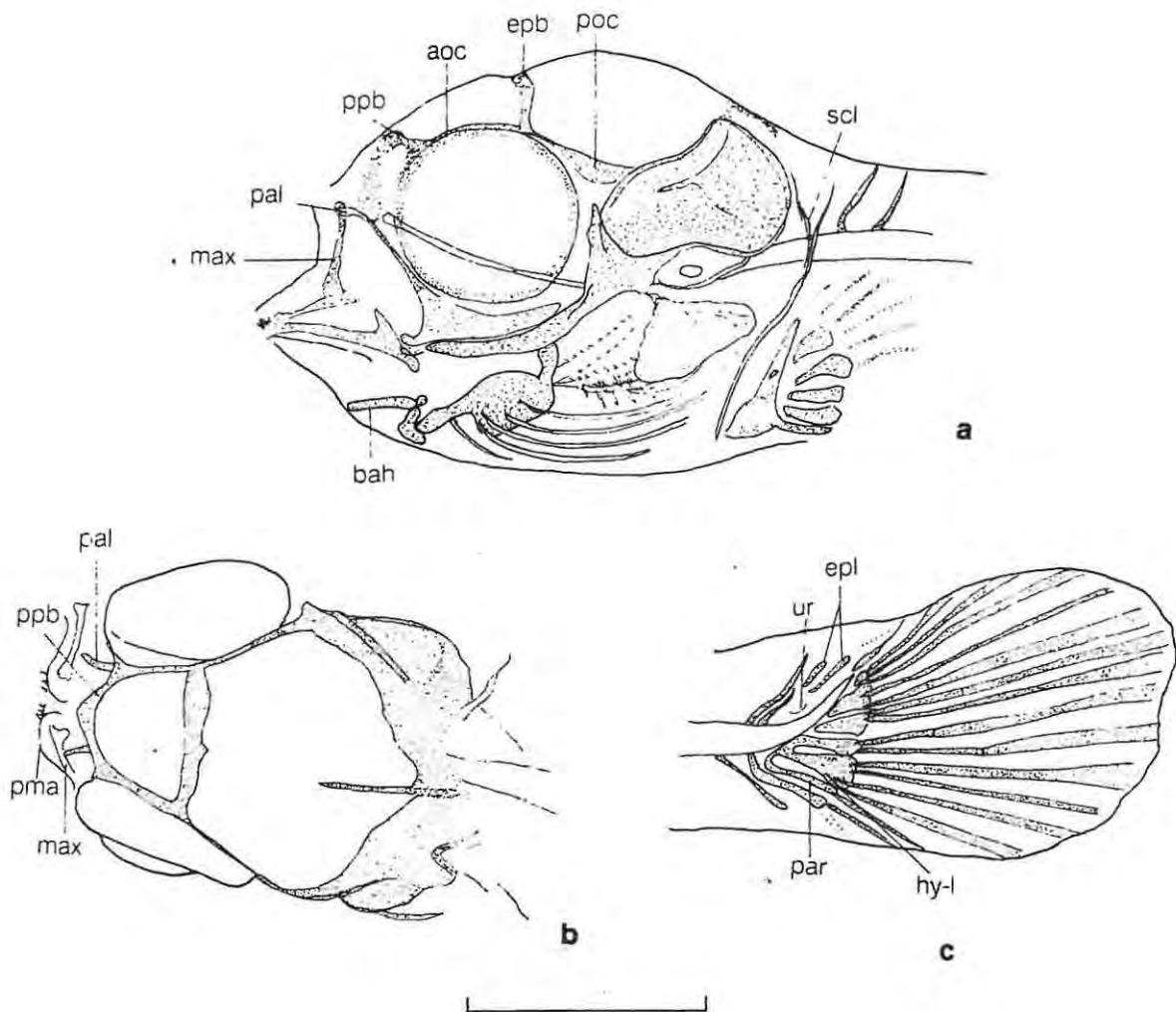
Figure 24 Step L¹10: Skeletal development at age 11:01 (TU = 276); a - lateral view of head, b - dorsal view of head, and c - lateral view caudal fin (aoc = anterior orbital commissure, bah = basihyal, epb = epiphyseal bar, epl = epural, hy-l = hypural 1, max = maxilla, pal = palatine, par = parhypural, pma = premaxilla, poc = posterior orbital commissure, ppb = paraphyseal bar, scl = sclera, ur = uroneural). Scale = 1.0 mm.

Figure 25 Step L¹10: Skeletal development at age 11:01 (TU =276). Calcified structures are not visible. Scale = 1.0 mm.

There was a marked increase in growth (4.1 mm) and a comparative decrease in yolksac area. These changes were particularly obvious when considering the absence of any changes in the previous step (Figures 9 & 19).

Step summary: By the end of this step all that remained of temporary embryonic structures was a remnant of yolk within the visceral cavity. All adult structures were present and functional except for the gonads. Skeletal development was predominantly in the structures relating to locomotion, thus increasing swimming efficiency for food acquisition and predator avoidance.

Release from the buccal cavity places the larvae into an environment where there is an increase in available oxygen. Mobility and the vitelline plexus would facilitate gaseous

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exchange. As a result the heartrate decreases. The quantity and quality of the exogenous food source coupled with the nutritional contribution of the large yolksac, allowed the rapid growth in length during this period. Increased morphological complexity was mainly in chondrification of feeding and locomotory structures.

Juvenile period ($J^1 11$) 15:00-?

The entire juvenile period was not documented but some information regarding the initial stages is presented below.

By age 15:00 (TU = 375) finfold differentiation was complete and the development of neuromasts and cupola along the lateral line extended up to and included the head region. Demarcation and ossification of individual vertebral centra was obvious (Figure 26). Spike-like processes had formed on the anterior and posterior corners of the vertebrae.

At age 15:12 (TU =388) the yolksac was visible through a narrow gap on the ventrum of the larvae (Figure 23b). In specimens from age 16:00 and older a large vein ran anteriorly along the dorsal surface of the spinal cord. This vein received blood from the caudal network and the intersegmental vessels. Many of the lepidotrichia in the tail had double loops and an intricate caudal pattern of crossing over and joining of vessels had developed. By age 16:03 (TU = 463), in most specimens, the opposing sides of the descending body wall tissue met, completely enclosing the visceral cavity and any remaining yolk (see Figure 23d). By age 18:12 blood vessels were noted at the bases of the dorsal and anal fins, and by age 19:12 (TU = 488) these vessels formed loops which extended partially up individual lepidotrichia. One row of scales had developed along the midline of the caudal peduncle. Over the next three days scales had extended anteriorly to the posterior margin of the operculum and

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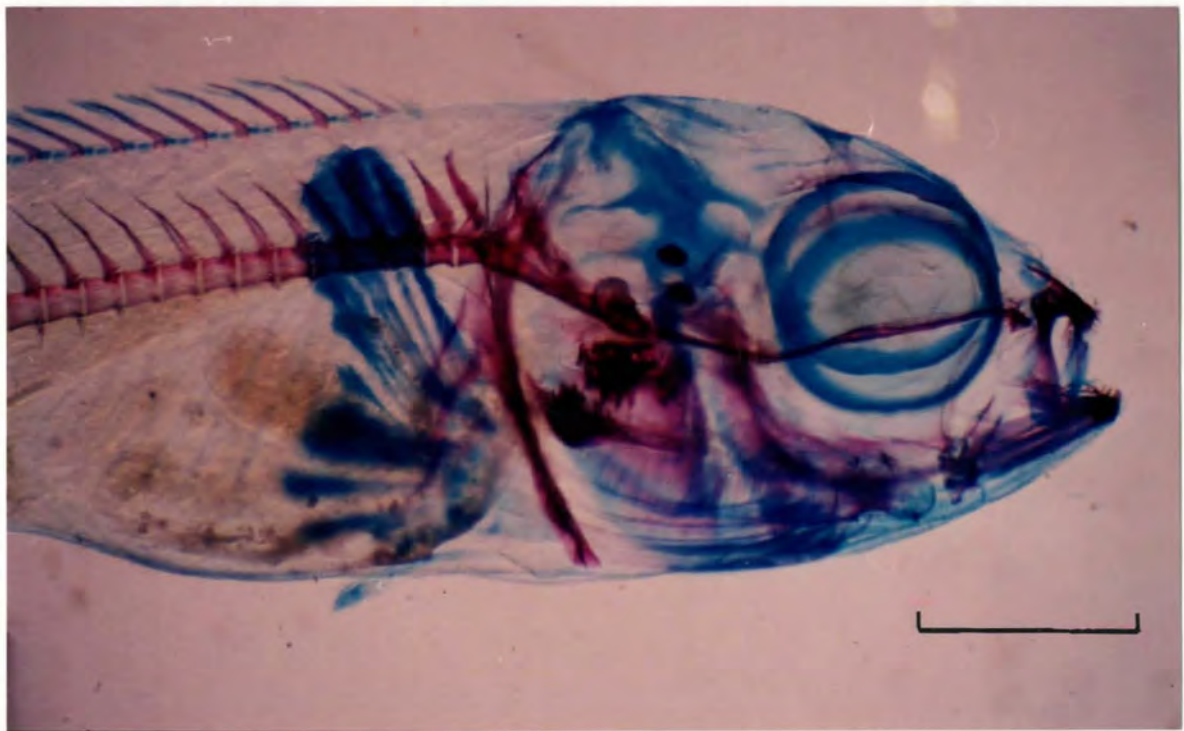
Figure 26 Step J^{1}11: Skeletal development of juveniles at age 15:00 (top) and at age 15:15 (bottom) illustrating the degree of calcification and the differentiation of the fins. Scales = 1.0 mm.

covered the trunk and tail region in all but the dorsal and ventral areas. Strong circulation existed in the both the median fins with the vessel loops extending along the lepidotrichia towards the distal fin margins. Pigmentation of the 'tilapia spot' was complete and created a rounded configuration and there were three faint vertical bars along the body, directly posterior to the head. Green and yellow melanophores and iridocytes were profuse over the entire body.

Variation

Development

Duplicate samples of specimens prior to hatching were difficult to assess for similarities in their level of development but no obvious, gross differences were detected. However, during the first few days there was a high rate of mortality and abnormalities. At age 03:09, an individual with a markedly higher heartrate was less developed than its sibling of the same age. Most samples up until age 09:04 had similar developmental rates and patterns. At that time, four embryos from the same clutch showed a gradient of differences from the smallest to the largest specimen (Figure 27). The ratio between yolksac size and embryonic length increased. In the smallest individual, the vitelline plexus consisted of a simple network of several large vessels. With an increase in embryo size, there was an increased complexity in vascularization as additional vessels had anastomosed, linking up the major vessels. The largest specimen had developed an intricate network of interconnections between slender, fine and large major veins, resulting in uniform vascularization over the entire yolksac surface. The larger specimen had a larger, vertically higher head, greater finfold differentiation, more profuse branchial vascularization and a larger visceral cavity. Pigmentation showed a lesser degree of difference. An outline of the major skeletal differences follows:

Largest specimen (7.0 mm): skeletal system - in general there was a greater degree of dechondrification and differentiation especially at articulation points; specifically - dentary and premaxilla teeth present, longer and more numerous pharyngeal teeth, maxilla slender and well-defined, five branchiostegal rays, scapula-coracoid slender and three well-defined components of the actinosal plate, fusion of hypural bases begun, posterior neural and haemal arches joined distally, three segments in the caudal lepidotrichia.

Smallest specimen (5.6 mm): skeletal system - no dentary or premaxilla teeth, four branchiostegal rays of a shorter length, two loosely defined actinosal plate components, maxilla broad and undifferentiated, hypurals free at bases, two segmented caudal lepidotrichia, neural and haemal arches shorter and none of them joined distally.

At age 11:01 another group of four individuals of the same parentage as the sample discussed above, manifested gross differences in their levels of development similar in magnitude to the embryos aged 09:04. Again, the largest and smallest specimens showed similar patterns of structural differences. The skeletal system is briefly outlined below.

Largest specimen (8.1 mm): skeletal system - generally most of the structures were present in both specimens but were markedly less differentiated and calcified in the smaller larvae; specifically - six premaxilla and dentary teeth, operculum larger, gill filaments on ceratobranchials, four radials defined from actinosal plate, pectoral rays present, all haemal arches joined distally, dorsal and anal fin pterygiophores and lepidotrichia present, 17 caudal fin rays with up to four segments and more defined, two epurals.

Smallest specimen (5.9 mm): skeletal system - two dentary and no premaxilla teeth, actinosal plate simple without radial

differentiation, only the last few haemal arches joined distally, no median fin development, central 16 caudal rays with three segments and marginal rays broad and indistinct, one epural.

Figure 27 Step $F^2$9: Four specimens from the same clutch at age 09:04 showing the degree of variation within a clutch. Scale = 1.0 mm.

The less advanced individuals of both ages discussed above, can not be compared to a level of development associated with younger specimens. The differences do not appear to be due to a general, overall slower rate of development. Possibly they are a result of an abnormality, a deformity or a deficiency related to their parentage.

Three individuals one hour younger (age 11:00) from a different clutch were all of a similar size and level of development (6.6, 6.9 & 7.1 mm) to each other and to the largest embryo aged 11:01 (described above). There was, therefore, intra-clutch but not inter-clutch variation in this instance.

There were slight degrees of variation in yolksac size, shape and enclosure, and in finfold differentiation in some individuals during the free-embryonic phase and the larval period. In the juvenile period, up to age 36:13, slightly darker pigmentation and tilapine spots were noted in two samples. These six differences were considered to be minor individual variations of no ontogenetic significance.

At age 36:13, of 11 individuals of the same clutch, one showed a retardation in development. The skeletal development was comparable to individuals from the early juvenile stages. The backbone was abnormally curved in the thoracic region. This deformity could possibly be attributed to its small size (11.5 mm as compared to the mean of 23.0 mm of the other individuals) and significantly lower level of development. Development of the remaining ten juveniles was comparable.

Total embryonic length, egg size and yolksac area
A one-way analysis of variance on the yolk areas followed by pairwise t-tests showed that the means of clutches 3 and 4 differed from the mean for clutch 6 but not from each other ($p \leq 0.016$). A multivariate analysis of minimum and maximum yolk lengths revealed that there was no significant differences over

time in maximum lengths within or between the clutches. However, significant differences in minimum length over time were found within clutches 3 and 4, and 4,2 (p = 0.033, 0.019, 0.040, respectively). Table 3 shows the results of a pairwise comparison of the means and the bivariate distribution of minimum and maximum egg length of all clutches.

Table 3 Results from pairwise comparisons between means and bivariate distribution of minimum and maximum egg length of <u>Oreochromis mossambicus</u> (ns = not significant, p = probably significant, s = significant).*

Clutch	3	4	4.2	6	6.2	6.3
3	–	ns	ns	s	s	p
4	ns	–	ns	p	s	s
4.2	ns	ns	–	ns	p	ns
6	s	p	ns	–	s	s
6.2	s	s	p	s	–	ns
6.3	p	s	ns	s	ns	–

*Because the data were used repeatedly between the pairs, the level of significance was set at 0.05/15 = 0.003. The p values marked as 'probably significant' were greater than 0.003 but were far below 0.05 and were therefore likely to be significant.

Maximum yolk length was constant over time but minimum yolk length was found to change with time within batches 3 and 4. A possible explanation for this inconsistency is that the measurements were inaccurate. The results of the yolk area analysis is consistent with the pairwise egg length analysis. Mean yolk area was similar between clutches 3 and 4, but was significantly less in clutch 6. Minimum and maximum egg length of clutch 6 was significantly smaller than all the other batches

except for clutch 4.2 (Table 3). Yolk lengths from clutch 6.2 and 6.3 were similar to each other but they were significantly larger than clutches 3 and 4.

The majority of embryonic length measurements were done on specimens from clutch 6, especially in the later stages of development, therefore, statistical clutch comparisons were not possible. In cases where there were similar aged specimens from different batches, no length differences were apparent. No relationship between parental origin or egg size can be deduced.

Heartrate

As mentioned in the descriptive text, heartrate throughout the embryonic and larval periods was erratic and inconsistent. Figure 28 shows the degree of fluctuation in the counts between individuals of differing ages and of similar aged individuals of the same or different parentage. Sample sizes were too small to draw conclusions about heartrates with respect to parental origin but a general pattern did emerge in some instances. The heartrates of clutch 6 specimens were usually higher and values for members of clutch 6.3 and 4.2 tended to be low.

Conclusions: Sample sizes were not sufficient to make any definitive remarks about inter- and intra-clutch variation. Relationships between development, egg size, embryonic growth and heartrate were not apparent except that clutch 6 had the smallest eggs and the highest heartrate.

The variation in sizes and levels of development between similar aged individuals from the same clutch is a common phenomena in all animal groups. One contributing factor could be egg size and yolk quality. Fishelson (1966) found that smaller eggs developed at a slower rate than larger eggs both inter- and intraspecifically. Further explanations are numerous and it is beyond the realm of this investigation to elucidate on the factors effecting variation amongst siblings.
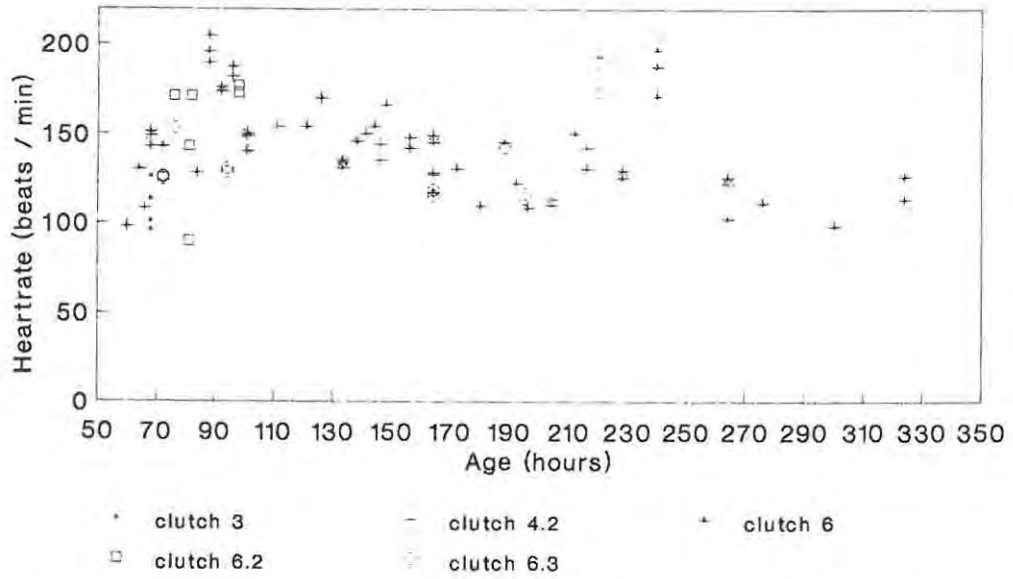
Figure 28 Fluctuations in heartrate of similar aged individuals within and between clutches.

CHAPTER 4: DISCUSSION

Periods in the early life-history of Oreochromis mossambicus

The results of this study suggest that the early ontogeny of O.
mossambicus consists of an embryonic period of 11 days and a
truncated or vestigial larval period of about four to five days.
The embryonic period is divided into a cleavage phase (1 day,
11 hours), an embryonic phase (3 days, 5 hours) and a free-
swimming embryonic phase (6 days, 8 hours). The juvenile period
begins at 15 days. Table 1 summarizes the ontogenetic events
of the steps within the phases and periods from activation until
juvenilization.

During the embryonic period the main or exclusive source of
nutrition is endogenous and the onset of exogenous feeding marks
the beginning of the larval period (Balon 1981, 1984b, Noakes &
Balon 1982, Flegler-Balon 1989). The larval period often begins
with an interval of mixed nutrition and continues to
metamorphosis, when most of the significant temporary embryonic
or larval structures are replaced with permanent adult
structures. Two of the most important morphological features
marking the end of the larval period and the beginning of the
juvenile period are the ossification of the axial skeleton and
the complete differentiation of the embryonic finfolds. With
fishes, an increase in the endogenous food supply and parental
care allows direct development of permanent organs, thus
bypassing the remodelling of temporary larval structures. In
direct development, the boundary between the embryonic and
juvenile period is the onset of exogenous feeding.   In some
cases, the interval of mixed nutrition and the persistence of
some temporary organs or structures is of a short duration,
resulting in a truncated larval period and a vestigial larva.
The larval period is often eliminated. In mouthbrooders, when
hatching or release coincides with the onset of exogenous
feeding, this marks a direct switch from the embryonic into the
juvenile period (Noakes & Balon 1982). The young are released

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in an advanced state of development and are considered to be juveniles.

Table 1 Step summaries of the early ontogeny of <u>O. mossambicus</u>.

Step	Age (days:hours)	Step summary
$C^1 1$	00:00-00:01	Bipolar differentiation; perivitelline space formation; hardening of egg envelope
$C^2 2$	00:01-00:22	Cleavage; germ ring formation; flattening of blastodisc
$C^3 3$	00:22-01:11	Gastrulation; beginning of epiboly; formation of embryonic shield
$E^1 4$	01:11-02:12	Neurulation; organogenesis; formation of fore-, mid- and hindbrain, optic vesicles, at least 17 pairs of somites, presumptive pericardial cavity and neuromeres; first pigmentation
$E^2 5$	02:12-03:00	First cardiac and muscle contractions; initial blood flow; formation of eye lenses, otic capsules, and heart-tube; simple vascularization of the vitelline plexus - anterior vitelline veins, blood islands; hemispherical division of the brain; elongation and separation of the tail
$E^3 6$	03:00-03:20	Strong head and body circulation; branching of the posterior vitelline vein; twisting of the heart-tube; some haemoglobin; first eye pigmentation; simple median finfold respiratory plexus; hatching begins
$E^4 7$	03:20-04:16	Hepatic vitelline respiratory network and branchial arteries develop; arterial flow into the preanal plexus; pectoral anlagen; hatching of almost all individuals
$F^1 8$	04:16-08:04	Formation of caudal finfold respiratory network, intersegmental and pectoral fin vessels and heart chambers; blood vessels in gill filaments; maximization of median finfold and vitelline respiratory plexi; anastomoses of profundal caudal vein for replacment of the inferior caudal vein; decline of median finfold respiratory network; head free of yolksac; chondrification of skeletal structures; beginning of dermal bone formation; peristalsis; jaw and pectoral fin movements; differentiation of stomach, spleen, and gall-, urinary- and swimbladder; beginning finfold differentiation; first iridocytes in eyes and retinal pigments
$F^2 9$	08:04-11:00	Replacement of the inferior caudal vein by the profundal caudal vein; disappearance of median finfold respiratory network; marked decline in vitelline respiratory plexus; formation and vascularization of secondary gill lamellae; increased formation of dermal bones; fusion of neural and haemal arches; formation of caudal lepidotrichia and proximal pterygiophores of the median fins
$L^1 10$	11:00-15:00	Mixed exogenous and endogenous feeding; ossification of chondroid bone, median, caudal and pectoral lepidotrichia; formation of vertebral rings and pelvic fin buds; filling of swimbladder; finfold differentiation and enclosure of yolksac almost complete
$J^1 11$	15:00-?	Finfold differentiation and yolksac enclosure complete; ossification of vertebrae; vascularization of dorsal and anal fins; squamation

Field observations done by Bruton & Boltt (1975) revealed that the young left the mouth of the female after age 07:00-08:00 and swam rapidly back when danger threatened. The level of development of the embryos aged 07:00-08:00 in this study, was not advanced enough to leave and return to the female's buccal cavity (see Figures 13-15). According to Vaas & Hofstede (1952) and Bohrer (1953) the time of first release for O. mossambicus is between ages 11:00 and 14:00. The earlier age coincides with my observations of the onset of exogenous feeding at age 11:00 (see Figure 21). At this age the young are well-advanced but cannot be considered juveniles based on the criteria of structure and organ development. Finfold differentiation and axial skeleton development had barely begun and were far from complete, and the yolksac was large and was the main source of nutrition. Although the temporary respiratory organs in the median finfolds had disappeared and the switch to branchial respiration had occurred, the vitelline plexus was still profuse and contributed to oxygen uptake. Between ages 14:00 and 15:00, finfold differentiation was completed, ossification of all the vertebra was well-established (though not always complete), the vitelline plexus had disappeared and the yolksac consisted of a remnant of yolk within the peritoneum (see Figures 21 & 22). These four developmental events coincide closely with both the oldest age of first release and the earliest age of final release (age 14:00) as suggested by Vaas & Hofstede (1952).

The ages of first and final release are not available for the fish used in this study, so all possibilities need to be considered. If first release had occurred prior to age 14:00, the embryos would not have been sufficiently advanced in their development to be considered juveniles, as discussed above. If they were released at age 14:00, they probably would not have been at the same level of development. This assumption is based on food acquisition and requirements. Although exogenous feeding may have occurred in the buccal cavity of the female, there was probably not sufficient quantity and quality of food for rapid growth and development. Crowded conditions and churning of the

embryos would exacerbate the situation. Released, free-swimming larvae would be more capable of acquiring larger quantities of food, thus facilitating rapid growth and development. First exogenous feeding occurred at age 11:00 and could coincide with first release. Skeletal development allowed clumsy swimming in most of the larvae and it was not for another 24 hours that efficient swimming abilities were observed in all the young. Although the larvae at the time of first release may be capable of some foraging and predator avoidance, the fact that the parent recalls the young suggests that the young are not developed enough to be fully independent. Without the parental care, the survival rate of the larvae would be greatly reduced.

The age of final release, between ages 14:00 and 22:00, coincides closely with the transition into the juvenile period on an eco-morphological and an eco-ethological level. In the case of O. mossambicus, and possibly other mouthbrooding cichlids with a recall of the young, if the time of release is used to determine the beginning of the juvenile period, the age at final release, not first release, is a more appropriate criterion. The interval between the age of first release coincidental with first exogenous feeding and the age of final release can thus be considered a truncated larval period. The larvae are, therefore, vestigial. This does not preclude the fact that the onset of the juvenile period may occur before final release. Ontogenetic development should be the criteria for the beginning of juvenilization and not parental care. In this study, a prolongation of the mouthbrooding period beyond 15 days would have been an example of overlap between juvenilization and final release.

The larvae of O. mossambicus do not develop any temporary larval features that undergo remodelling into adult structures during metamorphosis. Development from the embryonic condition directly into the adult condition while still feeding endogenously seems to be characteristic of this species. Therefore, it could be argued that there is no larval period in their early ontogeny.

In other words, there is no larval period based on ontogenetic criteria, but based on eco-morphological and eco-ethological parameters, there is. At first release the young are not embryos because their main source of nutrition is still endogenous. They are also not larvae because of a lack of specific, temporary larval structures, and they are not juveniles because they are still feeding endogenously and there is incomplete development of certain adult structures. This further supports my argument that there is a truncated larval period.

The evolutionary trend in fishes is towards a more specialized, precocial reproductive style (Balon 1985). Likewise, in anuran amphibians, reproductive modes exhibiting an advanced character state (apomorphic) were evolutionarily derived from primitive character states (plesiomorphic) (Duellman 1985, 1989). Altricial species characteristically produce numerous small eggs with low quality yolk. The embryos hatch at an early stage in their development and require an extended larval period (i.e. indirect development) for growth and/or dispersal and there is little or no parental investment in the form of hiding or guarding the eggs, embryos or larvae. With precocial species the eggs are fewer and the yolk is of a higher quality. The embryos hatch or are born in a more advanced stage of development and parental care and investment is extensive. The larval period is reduced or eliminated (i.e. direct development). The advanced developmental state at first release, the accelerated rate of development and brevity of the transitory larval period, and the intensive parental care and investment, place O. mossambicus at the precocial end of this evolutionary trend.

The ecological groupings of fish into reproductive guilds proposed by Kryzhanovsky were expanded by Balon (1981, 1990) to include more characters in an attempt to incorporate evolutionary patterns and relationships, especially during early ontogeny. This evolutionary classification includes three ethological sections of increasing specialization from nonguarders to guarders to bearers. These three sections are further

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partitioned into ecological groupings which are also arranged hierarchically according to the degree of protection allotted to the early life-history stages. Protection does not imply only parental care with respect to hiding, nest-building or bearing but can include increased endogenous food sources, leading to a more advanced level of development at first exogenous feeding, and direct development (Balon 1990). The truncated larval period of O. mossambicus implies an evolutionary trend towards direct development which is common in mouthbrooders and within the Cichlidae (Noakes & Balon 1982). According to Balon (1990) O. mossambicus is in the reproductive guild C.1.3, i.e. mouth brooders without buccal feeding. Within the Cichlidae, this species lies between the hole nesters, Tilapia rendalli, and the mouth brooders with buccal feeding, e.g. Cyphotilapia frontosa.

Saltatory ontogeny and homeorhetic states

The saltatory model is a useful tool in organizing the voluminous and complicated data which are generated from this type of research. It allows the researcher to order the events of development into natural, meaningful intervals rather than arbitrary stages. It is clear from my analysis that the components of a particular system or systems do synchronize their ability to function. For example, in the free-embryonic phase, one does not find a fully developed and functional gall bladder before the differentiation of the stomach, spleen and intestine have occurred. Likewise, prior to the switch to branchial respiration, there is not only synchronization between increased vascularization of the adult structures and the decline of the temporary embryonic structures, but muscular development in the ventricle ensures a strong flow of blood to the respiratory apparatus. Furthermore, these points of synchronicity occur just prior to a shift in form or function, be it internal or external, necessary for the next process to continue. The intervals leading up to a switch are homeorhetic states in that they are dynamic but stabilized states capable of maintaining and, concurrently,

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preparing for a change in the status quo. That change involves a shorter interval of instability which appears to occur rapidly considering the time it has taken the participating structures to reach that point of change. Characteristically, these intervals of rapid changeover are usually unstable periods of high risk for the organism, thus making it highly vulnerable. The above examples of synchronicity in the digestive, respiratory and circulatory systems occur prior to functional switches from embryonic to adult respiration, and from endogenous to mixed feeding. These transitions occur at time of first release, which puts the embryos at risk. Obviously, this includes a marked change in environment and the life-history of the fish.

During the cleavage phase in the ontogeny of O. mossambicus, the steps occurred rapidly, showed little variation and the boundaries were relatively easy to define. With later steps, it became increasingly more difficult to define boundaries. This can be attributed to three factors: morphological complexity, individual variation, and research design. The hierarchical complexity in morphology, from individual cells through groups of cells to tissues, organs and, eventually, organ-systems, makes it increasingly more difficult to ascertain interactive ontogenetic events and points of functional completion.

Shifts in the internal environment occurring on a lower level of resolution, such as cellular and enzymatic interactions and metabolism, were not investigated. Therefore, functional completion was based on morphological and behavioural criteria and changes in external environmental conditions. Obviously this was restrictive in determining the exact time of functionality and, consequently, threshold boundaries.

In O. mossambicus, individual variation was detected shortly after the onset of blood circulation (these differences may have been due to teratological reasons and not variation). Major variations in the level of development occurred between ages 09:04 and 11:01. The latter age coincides with the transition

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to mixed feeding and first release. Only minor differences were noted in older individuals. Similarly, in the diamond killifish Adinia xenica (Cunningham & Balon 1985) and the green sunfish Lepomis cyanellus (Champion & Whitt 1976, Shaklee & Whitt 1977) individual variation in morphological features and levels of enzymatic activity, respectively, was greatest between the onset of circulation and active swimming and feeding. Cunningham & Balon (1985) proposed that cumulative effects in individual variation may alter or shift events and thresholds. As a result individuals would develop their own ontogenetic pattern and make threshold distinction difficult. Some variation in rates and patterns of development can be expected but the consequences on thresholds, whether of minor or major importance, cannot be assessed from this study.

I am not certain whether development occurs in a saltatory fashion (as proposed by Balon 1984a, 1989) or gradually but at differing rates giving the appearance of saltation (Greenwood 1989). The point of contention is not whether ontogeny is sequential or synchronized, or whether there are boundaries separating important ontogenetic events. The disagreement arises over whether the nature of the changes from one level to another are saltatory.

Allowing for the difficulties encountered in stating the exact time of an ontogenetic event which constitutes a threshold, it appears that early ontogeny is saltatory. Whether the saltations occur at step or phase boundaries is not clearly evident. At period boundaries, especially those involving ecomorphological or eco-ethological changes in the life-history of the animal, there is a rapid change from one qualitative level to another. In O. mossambicus three definite steps exist. During the cleavage and embryonic phases there is a gradual increase in embryo length and a decrease in yolksac area (Figures 9 & 19). During the same time, the heartrate also increases gradually and peaks at the boundary between the embryonic and free-embryonic phase (Figures 10 & 20). During the free-embryonic phase, growth

and yolksac area depletion show little change. Initially, there is a gradual decrease in heartrate followed by a second peak at the boundary between the embryonic and larval periods. During the larval period, there is rapid growth and yolksac decline. At the same time, the heartrate has levelled off. Thus the cleavage and embryonic phases could be considered to be energetically expensive, the free-embryonic energetically cheap and the larval period, again, energetically expensive. The most obvious threshold is between the free-embryonic phase (embryonic period) and the larval period. Under natural conditions, the time spent in the buccal cavity of the female is an ecomorphological state with certain trophic attributes. The morphological and physiological features of the embryos are adapted to the conditions in the buccal cavity but they gradually undergo the necessary changes for the rapid shift in trophic attributes that will occur at first release. The coincidental event of first exogenous feeding makes this transition even more marked and in some ways is comparable to hatching in altricial fish.

Matsuoka (1987) proposed that there are saltatory qualitative changes in osteological and muscular development of the red sea bream, Pagrus major. The threshold boundaries occurred between the prelarval phase (a free-embryo phase in the embryonic period) and the larval period and between the larval and the juvenile periods. In the early ontogeny of a typical anuran tadpole, there are five stabilized states. However, the changes occurring in these 'stabilized states' are gradual (Duellman 1989). Duellman concluded that... 'the concept of saltatory ontogeny is applicable to progressive ecomorphological states in the ontogeny of an individual. These states are recognized by different morphological features and different trophic attributes, but the development of the morphological features and physiological attributes, which allow for changes in the trophic attributes, is gradual. This gradualism may exhibit plateaus, and the development of a given structure or attribute may be relatively slow or quite rapid.' Are the three most obvious steps in the ontogeny of O. mossambicus as discussed above, not the stabilized

states which exhibit plateaus as suggested by Duellman (1989)?

External environmental co-evolution and adaptations

The ability of this species to cope with three major
environmental influences (oxygen availability, predation pressure
and nutrition) are reflected in both its reproductive and
developmental strategies. Assuming that first release happens
at about age 11:00, the entire embryonic period of O. mossambicus
occurs while the embryos are within the buccal cavity of the
female, which effectively removes the problem of predation
pressure. The eggs are presumably highly nutritious due to their
large size and the density of the yolk, therefore, nutritional
requirements likewise pose no obstacle for the offspring.
However, the crowded and assumed low oxygen conditions within the
female's mouth have made it necessary for the embryos to develop
a profuse and complex temporary respiratory organ on the ventral
finfolds and the yolksac. Oxygen uptake may be facilitated by the
flapping of the pectoral fins which creates a water current over
the vitelline plexus. Fishelson (1966) equated the pectoral fin
movement of mouthbrooding larvae with that of the tail
'wriggling' of substrate spawners and concluded that it has a
respiratory function.

During the larval period, a new and different situation arises.
Oxygen is not a problem for the newly-released, mobile larvae in
shallow, sandy, wave-washed nursery areas where oxygen levels are
high. In habitats of dense vegetation, oxygen availability could
be low, especially at night, but this deficiency is overcome by
the respiratory function of the large, well-vascularized yolksac.
Mobility and pectoral fin movements would facilitate oxygen
uptake. The yolksac serves a secondary purpose of supplying
sufficient nutrition until the period of mixed feeding ends. The
nurseries of O. mossambicus are commonly in well-vegetated areas
that offer an abundance of food for the larvae. Predation,
however, is a serious consideration at this time and three

adaptive features have evolved to overcome this threat. Firstly, during most, if not all, of the larval period, there is recall of the offspring into the female's buccal cavity whenever a threatening situation arises. Secondly, the level of development of the larvae is relatively well-advanced at this stage and consequently so is their ability to avoid predators. And finally, the plants in the nurseries offer cover and camouflage.

The co-evolution of this species and its external environment is clearly reflected in the ecomorphological and eco-ethological characteristics of its reproductive and developmental life-history style. The ecomorphological contributions from the adult include an egg with a high carotenoid and nutritional content for increased utilization of oxygen and rapid development and growth, respectively (see Balon 1977, 1979 & 1984b for information about the oxidative role of carotenoids). Eco-ethologically, the act and duration of mouthbrooding, release and recall, and the ability of the mother to move into appropriate nursery areas is an adaptation to predatory, nutritive and oxygen parameters. As regards the offspring, ecomorphological adaptations include the presence of well-developed temporary respiratory organs to cope with low oxygen levels in the buccal cavity. Overall rapid development is advantageous. The skeletal and the sensory systems facilitate exogenous food acquisition and prey avoidance. Pigmentation assures camouflage. The circulatory and respiratory systems are vital for the assimilation of nutrients and growth. The truncated larval period and interval of mixed feeding are both adaptations for protection of the young (Noakes & Balon 1982, Balon 1984b).

Phenotypic plasticity in the early life-history style and the reproductive guild of O. mossambicus is an important factor in the ability of this species to colonize and invade different habitats. Their invasive capabilities go beyond successful translocations in southern Africa (de Moor & Bruton 1988) as they are internationally one of the most successful natural and man-aided fish invaders (Bruton 1986). The specific early life-

history traits which exhibit a high level of plasticity that allows such successful invasion are numerous. The variable release time allows an extended interval of parental care which could increase the duration of benign conditions that mouthbrooding provides. On the other hand, if environmental conditions are unsuitable, the female could tend the young until better conditions exist. Another alternative would be for her to move to a more suitable habitat. The persistent vitelline plexus supplements the nutritional and oxygen requirements of the newly released larvae. This is of particular importance in sub-optimal habitats and could result in an extended larval period. Conversely, in optimal conditions the larval period could be shortened. These heterochronic shifts in duration or timing of ontogenetic events not only allow increased plasticity for the individual or the population, but can alter the life-history styles of successive generations if the shifts are persistent over time. Heterochronic shifts due to environmental influences are an important factor in the formation of an ecophenotype (Balon 1985).

Comparisons with other ontogenetic studies done on O. mossambicus and other Cichlidae

Numerous studies have been conducted on the early life stages of cichlid fish but comparisons are difficult due to differing temperatures, techniques, terminology and motivations. The objectives are often to describe a specific structure (i.e. temporary respiratory structures, Lanzing 1976) or interval in the embryonic or larval period (i.e. transition to active feeding, Bogdanova 1970). The most comprehensive work on cichlids was done by Fishelson (1966) and Balon (1977).

Chacko & Krishnamurthi (1954) and Bogdanova (1970) briefly described early stages in the life-history of O. mossambicus (Tilapia mossambica in their papers). Although there was no reference to temperature regime in the former study and the

sampling regime in both cases was not as specific as that used in this study, some of their findings were comparable with those of this project. For example, hatching commenced at the end of the fourth day (Chacko & Krishnamurthi 1954) and the larvae began exogenous feeding six days later, that is approximately 11 days after fertilization. Bogdanova (1970) observed that larvae began feeding seven days after hatching. The hatching time was not reported but assuming it was similar to that observed in this study, first exogenous feeding probably occurred at the same time as that reported by Chacko & Krishnamurthi (1954). In this study hatching began 3 days and 15 hours after activation, and by the end of the fourth day almost all the embryos had hatched. First exogenous feeding occurred between 7 and 8 days later (approximately 11 days after activation). Direct and exacting comparisons can not be made between these three studies but the rate and pattern of some of the early ontogenetic events showed a great deal of overlap and similarity. Despite the lack of information about, and the differences in, experimental conditions of the above comparison, it appears that the early development of O. mossambicus is relatively consistent when the fish are reared in artificial habitats, at least with regards to hatching and first exogenous feeding. However, a large discrepancy in the time of yolksac enclosure exists between Fishelson's (1966) and my observations (Tilapia mossambica in his paper). According to his findings, the yolksac was enclosed by the body wall between 8 to 10 days (TU = 216-270). In my study, similar aged individuals (TU = 200-250) still had a large yolksac and migration of the body wall over the yolksac was only partial (see Figures 14 & 16). Complete enclosure occurred between 16 and 17 days (TU = 403- 425) (see Figure 23). Possible but unlikely explanations for this discrepancy are temperature, egg size and interpretation differences. A temperature difference of 2°C between the two studies would not account for the diverse observations. Even with a difference of 4°C, Shaw & Aronson (1954) found a maximum temporal difference of three days between ontogenetic events of S. melanotheron (Tilapia macrocephala in their paper). Yolksac enclosure, as I use it,

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refers to the meeting of the descending body wall as it migrates over the yolksac, in which case there is some yolk remnant present in the visceral cavity. Some authors expand on the above definition to include the total absorption and assimilation of all the yolk. It is not clear to me which of the above terms applies to Fishelson's observations but if he was using the latter definition, the discrepancy between his and my observations would be even greater. Developmental rates are faster in small eggs and slower in larger eggs and the variation within mouthbrooding fish species is greater than in substrate spawning species due to the larger variation in egg size (Fishelson 1966). The egg sizes of the O. mossambicus in Fishelson's findings were 1.9-2.6 mm without the membrane. In this study, the eggs measured 2.8-3.2 mm with the membrane. Even accounting for the additional length due to the membrane, my fish eggs were larger but the egg size differences are probably not sufficient to explain the differing times of yolksac enclosure.

Development prior to somite formation was similar between O. mossambicus, Oreochromis niloticus (Galman & Avtalion 1989) and Labeotropheus sp. (Balon 1977). Table 2 compares the timing and pattern of ontogenetic events after somite formation of four species of mouthbrooding cichlids, O. mossambicus, O. niloticus ('Tilapia nilotica'), Sarotherodon melanotheron ('Tilapia macrocephala') and Labeotropheus sp. and one substrate spawning cichlid, Tilapia tholloni. In the case of O. niloticus, two sources of information are included. The ages listed refer to the first occurrence of an event. All of the experiments were conducted under artificial conditions at a temperature of ±25°C, except for Fishelson (1966), who reared his fish at ±27°C. Discrepancies were noted in the timing of some events when consulting the text, the tables and the summarizing graph in Fishelson's paper. In these cases, I consistently used the tabular information for my comparisons. An attempt to include events of ontogenetic importance was made but this was not always possible due to differences in: a) sampling frequency and time, b) levels of resolution, c) detail of the description, and, d)

authors' biases about what structures to include in the description or their interpretation of the level of development. An additional restriction was my interpretation of their interpretation.

T. tholloni showed the greatest differences in the timing of events when compared to _O. mossambicus_ (Table 2). Out of a total of 35 characters, the development was considerably faster in all but four cases (segmental vessels, dentary teeth, urostyle ossified and concave vertebral centra). In these four instances, the development was slower in the substrate spawner than in all the mouthbrooding species (except for _Labeotropheus sp_). In mouthbrooders (_O. mossambicus_ included) the profundal caudal vein develops from posterior segmental veins (Fishelson 1966). This explains why the segmental vessels occur earlier in the ontogeny of the mouthbrooders. Fishelson also found that the rate of organogenesis of the axial skeleton decreases in substrate spawners after yolksac enclosure, thus the temporal differences in the ossification of the urostyle and the concavity of the vertebral centra.

With _O. niloticus_ (Fishelson 1966) the developmental rate of all 18 compared characters was faster than with _O. mossambicus_. Of the 17 characters compared with _O. niloticus_ (Galman & Avtalion 1989) 11 developed faster, five were slower and one was comparable (Table 2).

With _S. melanotheron_, 24 of the characters occurred at an earlier age, four were at an older age and six were comparable to _O. mossambicus_ (Table 2). By comparing temperature units, the situation changes slightly. Fifteen events occurred earlier, six were later and nine were comparable. In all but one instance, the differences were greater between _O. mossambicus_ and _O. niloticus_ than they were with _S. melanotheron_.

Table 2 Comparisons of the timing of ontogenetic events of <u>Oreochromis mossambicus</u>, <u>Oreochromis niloticus</u>*, <u>Sarotherodon melanotheron</u>*, <u>Labeotropheus</u> sp. and <u>Tilapia tholloni</u>. The ages listed refer to the first occurrence of the event. Numbers in brackets below the species names refer to the sources of information and the figures in parenthesis following the ages refers to temperature units. Direct comparisons can be drawn between results of this study and sources 1 and 3 due to similar temperature regimes (done at $\pm 25^\circ$C). Dotted lines represent a step boundary. 1 = Galman & Avtalion 1989, 2 = Fishelson 1966, 3 = Balon 1977. (*'<u>Tilapia nilotica</u>' and '<u>Tilapia macrocephala</u>' in Fishelson 1966).

Ontogenetic event	O. mossambicus	O. niloticus (1)	O. niloticus (2)	S. melanotheron (2)	Labeotropheus (3)	T. tholloni (2)
Somites 9-12	01:19 (45)		01:04(29)	01:10 (38)	02:07 (57)	00:20 (23)
Pigmentation on yolksac	01:21 (47)	02:04 (54)			02:03 (53)	
Optic vesicles	02:00 (50)	02:04 (54)	01:10 (35)	01:16 (45)	02:01 (51)	00:21 (24)
Somites-16-18	02:03 (53)			01:22 (52)	02:22 (73)	01:05 (33)
Brain 3 segments	02:03 (53)	02:10 (60)	01:00 (27)	01:16 (45)	02:03 (53)	00:18 (20)
Eye lenses	02:12 (63)	02:17 (68)		03:08 (90)	02:10 (60)	02:00 (54)
Heartbeats	02:12 (63)	03:00 (75)	01:12 (41)	02:00 (54)	02:16 (67)	01:00 (27)
Body movement	02:16 (67)		01:12 (41)	01:22 (52)	02:16 (67)	00:23 (26)
2 otoliths	02:20 (71)		02:00 (64)	02:15 (71)	02:10 (60)	01:12 (41)
Eye pigmentation	03:10 (85)	02:17 (68)	02:04 (58)	03:00 (81)	03:20 (96)	01:20 (50)
Finfold plexus	03:14 (90)		02:20 (77)	03:08 (90)	05:17 (143)	01:18 (47)
Hatching begins	03:15 (91)	03:06 (75)	02:22 (79)	03:22 (106)	05:14 (140)	01:22 (52)
Hepatic/mesenteric vitelline system	03:20 (96)			04:12 (122)	06:02 (152)	
Pectoral anlagen	03:20 (96)	03:06 (75)			05:16 (142)	
Gill circulation	03:22 (98)				04:00 (100)	01:22 (52)
Segmental vessels	04:16 (117)		03:08 (86)	04:00 (108)	05:14 (140)	04:22 (133)
Urostylar vessels	04:16 (117)			06:00 (162)	06:02 (152)	04:00 (108)
Nasal canal open	04:20 (121)	04:02 (102)			04:21 (122)	
Chondrification in suspensorium	05:13 (139)			05:00 (135)		03:00 (81)
Maximum finfold plexus	05:13 (139)			<05:00 (135)	07:02 (177)	05:00 (135)
Eyes dark	05:13 (139)	03:06 (75)			05:14 (140)	
Yellow substance in gut	06:02 (152)			07:00 (189)		02:12 (68)
Mouth open	06:04 (155)	04:02 (102)		06:00 (162)	07:02 (177)	04:00 (108)
Gill filaments vascularized	06:12 (163)				08:02 (202)	06:00 (162)
Pectoral fins vascularized	06:20 (171)	06:20 (171)		07:00 (189)	07:02 (177)	04:00 (108)
Pectoral fins move	06:20 (171)			06:00 (162)	05:14 (140)	
Jaw movement	06:20 (171)	04:02 (102)			07:02 (177)	
Caudal fin rays	06:20 (171)	05:02 (127)	05:00 (135)	06:00 (162)		03:12 (95)
Pharyngeal teeth	06:20 (171)			07:00 (189)		04:12 (122)
Dentary teeth	07:12 (188)	06:20 (171)		07:00 (189)		08:00 (216)

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Table 2 continued

Ontogenetic event	Age days:hours (TU)					
	O. mossambicus	O. niloticus (1)	O. niloticus (2)	S. melanotheron (2)	Labeotropheus (3)	T. tholloni (2)
Primordial pro-fundal caudal vein	07:20 (188)				08:02 (202)	05:00 (135)
Anus open	08:00 (200)		06:12 (176)	07:12 (203)		04:12 (122)
Peristalsis	08:03 (203)		06:00 (162)	06:00 (162)		03:12 (95)
Inferior caudal vein gone	08:04 (204)				09:02 (227)	07:00 (189)
Ossification of branchiostegal rays	09:00 (225)			07:00 (189)	21:04 (529)	08:00 (216)
Median fin rays	10:00 (250)		06:00 (162)	07:00 (189)	10:02 (254)	05:00 (135)
First ossification in suspensorium	10:04 (254)			10:00 (270)	21:04 (529)	08:00 (216)
First exogenous feeding	11:00 (275)	10:16 (267)	09:00 (243)	11:00 (297)	25:00 (625)	06:00 (162)
Urostyle ossified	11:00 (275)		09:00 (243)	11:00 (297)	21:04 (529)	14:00 (378)
Vertebral rings	11:00 (275)		07:00 (189)	09:00 (243)	25:00 (625)	09:00 (243)
Concave vertebral centra	14:12 (363)			12:00 (324)		16:00 (432)
Yolksac enclosure	16:00 (403)	10:16 (267)	09:00 (243)	12:00 (324)	21:03 (528)	07:00 (189)
Median fin vascularization	19:12 (480)			15:00 (405)	11:02 (277)	

With Labeotropheus, the converse situation exists. Twenty-four of the characters developed slower, three were faster and eight were comparable to O. mossambicus (Table 2). A majority of the structures showing a slower developmental rate were related to the circulatory or respiratory systems. In the last nine characters, very marked differences in rates of development became apparent with respect to skeletal development and yolksac enclosure. This can be attributed to the longer incubation period of between 25 to 30 days (brooding at 25±0.5°C) of Labeotropheus (Balon 1977). Recall into the buccal cavity was not observed and the juveniles began foraging immediately after release. The level of skeletal development was comparable to that of O. mossambicus at age 11:00, the age of first exogenous feeding and presumably near first release. Labeotropheus, unlike O. mossambicus, resembled the adult phenotype and yolksac enclosure and finfold differentiation were complete.

With all four species, there were not any instances where the sequence of ontogenetic events was identical with that of O. mossambicus. The greatest and least degree of deviation was with T. tholloni and S. melanotheron, respectively.

There was overlap in the ranges of egg lengths of O. mossambicus with those of O. nilotica and S. melanotheron (Fishelson 1966). Labeotropheus and T. tholloni had larger and smaller eggs, respectively. Embryonic lengths of O. mossambicus did not show any similarities with any of the species, except that Labeotropheus embryos were slightly larger.

S. melanotheron development (Shaw & Aronson 1954) lagged behind that of O. mossambicus. All the comparable ontogenetic events showed large differences in timing. For example, at a temperature of 25°C hatching occurred on the tenth day as opposed to between the third and fourth day, as found with O. mossambicus.

To summarize, the early ontogeny of O. mossambicus was found to be intermediate between that of Labeotropheus sp. and S. melanotheron. O. mossambicus differed the most in rate and pattern of development with T. tholloni and the least with S. melanotheron. Generally, the developmental pattern was similar but with temporal differences when comparing all the species. The differences which did occur in the ontogeny were mainly between the substrate brooder and the mouthbrooders and can be attributed to the differences in their life-history styles and egg sizes. The ontogeny of the differing eco-ethological groups appears to be environmentally evolved for optimal use. Fishelson (1966) concluded that egg size and yolk supply influence developmental rates. He felt that the temporal differences in ontogeny of mouthbrooders was primarily a result of egg size, and that the phyletic relationships were a secondary factor. Based on egg size and phyletic relationships, the comparison provided by this study would have put the early ontogeny of O. mossambicus between that of O. niloticus and S. melanotheron. Furthermore,

there would have been a closer relationship between the cogenitors. A third factor to consider is the life-history style of the two genera. In both of the Oreochromis species, there is recall of the young into the buccal cavity of the female for about 2-3 weeks (Trewavas 1983), whereas with both S. melanotheron and Labeotropheus the young are released at a later age and there is no recall. I have no explanation for the position of O. mossambicus between S. melanotheron and Labeotropheus with regards to their ontogeny. Possibly the differences are a result of misinterpretation or differing experimental conditions. There may be other influencing factors that have not been considered, such as salinity, water condition and light regimes. A major drawback in all cases is that the fish were removed from their natural habitat and the complex interactions present in all populations, communities and ecosystems were absent. An important aspect of their life-history, the early ontogeny, was thus analyzed in isolation and the underlying contributing factors played no role.

In conclusion, the early ontogeny of O. mossambicus consists of an embryonic period with three phases lasting approximately 11 days and a truncated larval period of about 4-5 days. It seems likely that the entire embryonic period could occur while the embryos are in the female's buccal cavity and that first release may be coincidental with first exogenous feeding and the transition into the larval period. Recall of the young into the female's mouth may extend into the juvenile period. Further research, preferably in the field, will be necessary to confirm these speculations about the level of ontogeny during incubation.

There are boundaries and thresholds between important morphological, ontogenetic and ethological events during early ontogeny. Whether the changes at these boundaries are saltatory or gradual between steps within the phases and periods is not clear, but there is evidence that the shifts between the embryonic phase and the free-embryonic phase and between the

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free-embryonic phase (embryonic period) and the larval period are of a saltatory nature. All three of these important transitional events occur when the trophic features of the environment change, thus presenting the ebmryos/larvae with a new set of parameters to cope with and placing them at some risk.

Phenotypic and life-history plasticity, so well documented in the adult period of this species, is also characteristic of the embryonic and larval periods.

CHAPTER 5: REFERENCES CITED

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