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The chronological development of the urodele amphibian *Pleurodeles waltl* (Michah)

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Foreword

The Normal Table of Development of the urodele amphibian *Pleurodeles waltl* was published almost 40 years ago by Gallien and Durocher (1957) and provides a good description of the chronological development of this species. Unfortunately the journal in which the work was published had a very limited circulation and is now difficult to obtain. Gallien and Durocher's table remains valid since it provides both a precise timing and morphological criteria for *Pleurodeles* development. Our intention, as enthusiastically suggested by Dr. Juan Aréchaga, is to update this table by following the development of the embryo with light and electron microscopy photographs, which reproduce faithfully the embryo morphology. The staging remains as defined in Gallien and Durocher's table on the basis of development at 18°C, with the exception of the larval stages, which have been reexamined and restaged. Since laboratory temperature may vary, the times give only a rough indication of developmental speed.

Introduction

Pleurodeles eggs have proved to be useful for many aspects of cellular and molecular embryology studies. The eggs have a relatively slow development, allowing cellular movements to be followed easily *in vivo* and *in vitro*. In addition, recent work has revealed that *Pleurodeles* embryos differ from other amphibian species, such as the anuran *Xenopus laevis*, in the origin and arrangement of mesodermal cells (compare the fate maps of these two species in Fig. 1). Thus in the early *Pleurodeles* gastrula, cells from the outer- and innermost layers of the entire marginal zone contribute to mesodermal structures. As a consequence, mesodermal cell migration makes an important contribution to gastrulation in contrast to *Xenopus laevis*, in which convergent extension is the main driving force. After metamorphosis, the urodele also offers advantages for studying several aspects of morphogenesis. Overall, the rather different development of this species makes it interesting for comparative studies in developmental biology.

Cellular and molecular embryology studies require a precise understanding of the normal sequence of development because cell interaction and differentiation occur in a coordinated spatial and temporal pattern. In addition, many genes regulating early development have recently been identified, and typically show a temporal and spatial pattern of expression in the embryo. In order to understand the significance of these patterns it is important to be able to correlate them with morphogenetic events.

In general, early amphibian development is supported by maternally-derived mRNA (information stored in the oocytes). Zygotic transcription starts only at middle blastula stage, during the "mid-blastula transition" (MBT). The initiation of transcription concerns not only novel RNA species but also RNAs which were also present during the cleavage stages.

The amphibian oocyte has radial symmetry with its axis passing through the animal and vegetal poles. At the moment of fertilization cortical cytoplasmic rotation establishes the dorsal-ventral axis. Initially the egg can be considered to have two predetermined germ layers. When isolated and cultured *in vitro*, the animal hemisphere will give rise to ectodermal structure and the vegetal hemisphere will form endoderm. Mesoderm, which derives from a zone between the animal and vegetal regions called the marginal zone, appears to form as a result of interaction between the vegetal and animal cells during cleavage stages. The three germ layers are brought into place by the gastrulation movements, thus establishing the basic body plan. The mechanisms underlying gastrulation are fundamentally similar in various amphibian species, but the details vary considerably. Recent work using cell lineage tracers has defined the fate of different blastomeres and their contribution to various embryonic structures. In addition to the so-called "fate map" in various species generated by these techniques, a body of information on the timing of steps of progressive determination of cell fate has come from embryological manipulations such as microsurgical dissection of embryonic tissues and targeted gene expression.

Before gastrulation the egg has a relatively simple organization. The blastula consists of embryonic cells arranged around a central cavity called the blastocoel. There is a brown-pigmented animal cap composed of small blastomeres with relatively little yolk, and

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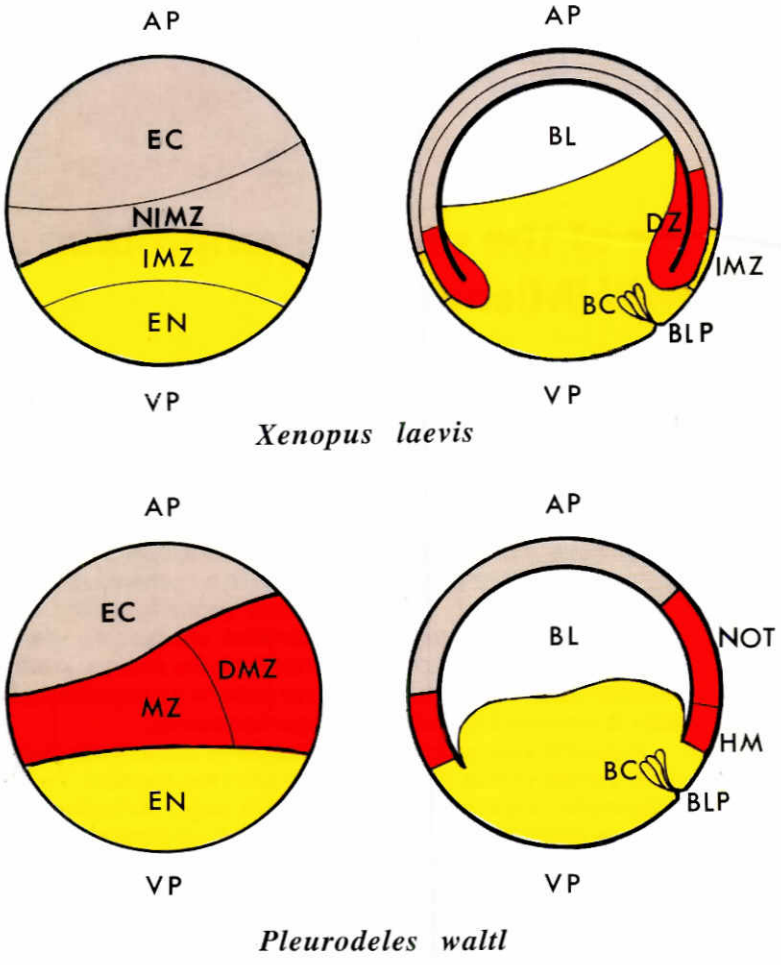

Fig. 1 . Fate maps of *Xenopus laevis* (from Gerhart and Keller, 1986) and *Pleurodeles waltl* embryos (from Nieuwkoop, 1985). *In Xenopus laevis early gastrula the superficial layer of the involuting marginal zone (IMZ) consists of endodermal cells, the mesoderm is located in the deep marginal zone (DZ), so that the surface of the embryo consists of ectoderm (EC) and endoderm (EN) cells. Both the non-involuting marginal zone (NIMZ) and the IMZ cells undergo extensive convergent extension during gastrulation. In Pleurodeles waltl early gastrula the superficial layer of the entire marginal zone (MZ) consists of mesoderm cells. The dorsal marginal zone (DMZ) cells migrate actively during gastrulation. AP, animal pole; VP, vegetal pole; BL, blastocoel; BLP, blastopore; BC, bottle cells; HM, head mesoderm; NOT, notochord.*

a vegetal region composed of large, pale, yolk-laden blastomeres. The marginal zone is composed of blastomeres of intermediate size, pigmentation, and yolkiness. The blastocoel roof consists of two layers of cells, and on its inner surface the extracellular matrix is formed. During gastrulation extensive cell movements occur in a coordinated manner. The initiation of gastrulation sets the orientation of the new embryonic axis. Both the dorso-ventral and antero-posterior axes are definitively established at this stage, and so the embryo acquires bilateral symmetry.

The initial step in gastrulation is the formation of the dorsal lip of the blastopore. The blastopore spreads laterally and ventrally from the dorsal lip to eventually form a crescent-shaped slit, and then a complete circle. A broad sheet of cells above the dorsal lip of the blastopore (the primordium of the chordamesoderm) moves toward, and over, the dorsal lip in a movement known as involution. Although several rather different mechanisms, such as epiboly and convergent extension, are involved in *Pleurodeles* gastrulation, it appears that migration of mesodermal cells is the most important. The arrangement of mesoderm in both the outer- and innermost layers of the marginal zone facilitates analysis of mesodermal cell migration.

As gastrulation proceeds, a new cavity, the archenteron, is formed as the invagination site at the blastopore deepens and expands toward the animal pole inside the embryo. The cells come to underlie the basal surface of the blastocoel roof and induce the overlying presumptive ectoderm to form the neural plate, which later develops into the central nervous system. Since in *Pleurodeles* the outermost cells of the dorsal marginal zone contribute to mesodermal structures, the archenteron roof is initially formed by chordamesodermal cells. The neural plate forms as a thickening of the dorsal ectoderm, which is an unicellular layer composed of columnar cells. The lateral borders elevate and converge to form the neural folds which eventually fuse at the dorsal midline. A neural tube is thus formed. At the same time endodermal cells on the lateral sides of the archenteron also converge dorsally and ultimately meet beneath the notochord, making the lining of the gut anlage. It is worth noting that the neural plate is much more enlarged in the head region than in the trunk region. The fusion of neural folds begins in the trunk region, then extends progressively to the anterior and posterior regions. At the same time the embryonic body elongates. At the tailbud stage, the principal organs are established and the tail elongates. All organs except the gonads become fully developed before metamorphosis. During the larval stages the *Pleurodeles* has three pairs of branching gills as the principal respiratory apparatus. These disappear gradually during metamorphosis.

For a detailed description of the histology of the embryo readers are invited to consult related books dealing with histogenesis and organogenesis. We give an external description of each stage and whenever possible, a brief internal description will be given, especially at the gastrula stage.

Acknowledgments

We are particularly grateful to Dr. J. Aréchaga for encouraging us to prepare this revised Table of Pleurodeles waltl development, without whose suggestion the work, though initiated almost 10 years ago, would not have been brought to light. We thank Dr. H. Boulekbache for giving us the opportunity to use the scanning electron microscopy, Dr. E. Houliston for careful reading of the manuscript, and J. Dérosiers for help with illustrations.

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Developmental stages of *Pleurodeles waltl* (Michah)

Cleavage stages

Stage 0. One-cell stage; 0 h; 1.7 mm. Animal hemisphere pigmented brown, while vegetal hemisphere appears clear green. Egg rotates about 1 h after fertilization. Maturation spot disappears at about 3 h and pigmentation in animal hemisphere is uniform. At 3 to 4 h, the future dorsal side becomes less pigmented, appearance of a lightly pigmented crescent. Egg surface covered by microvilli, especially in animal hemisphere.

Stage 1. 2-cell stage; 6.5 h; 1.7 mm. First plane of cleavage, dorsal side is further depigmented. Accumulation of microvilli in cleavage furrow. First plane of cleavage does not necessarily correspond to plane of bilateral symmetry.

Stage 2. 4-cell stage; 7.5 h; 1.7 mm. Second plane of cleavage divides egg into 4 nearly equal blastomeres. Dorsal side less pigmented than ventral region.

Stage 3. 8-cell stage; 9 h; 1.7 mm. Third plane of cleavage above the equatorial region. Formation of 4 microblastomeres in animal region and 4 macroblastomeres in vegetal region. Dorsal blastomeres less pigmented than ventral blastomeres.

Stage 4a. 16-cell stage; 10.5 h; 1.7 mm. Cleavage furrows first appear in animal microblastomeres, then in vegetal macroblastomeres.

Stage 4b. 32-cell stage; 12 h; 1.7 mm. Four tiers of 8 blastomeres roughly regular. Dorsal animal blastomeres still less pigmented than ventral animal ones.

Stage 5. Early blastula; 14 h; 1.7 mm. Blastomeres are still large in size. Difference in size between animal and vegetal blastomeres can be distinguished easily in lateral view. Difference in pigmentation between dorsal and ventral blastomeres has disappeared. Internally blastocoel cavity well developed.

Stage 6. Mid-blastula; 22 h; 1.7 mm. Intermediate blastomere size, gradual blastomere size difference observable from animal to vegetal blastomeres. Tangential division gives rise to 2 cell layers in blastocoel roof.

Stage 7. Late blastula; 27 h; 1.7 mm. Animal and vegetal blastomeres both smaller in size. Pigmentation roughly uniform in animal hemisphere. Blastocoel roof consists of 2 layers of cells. Beginning of zygotic transcription.

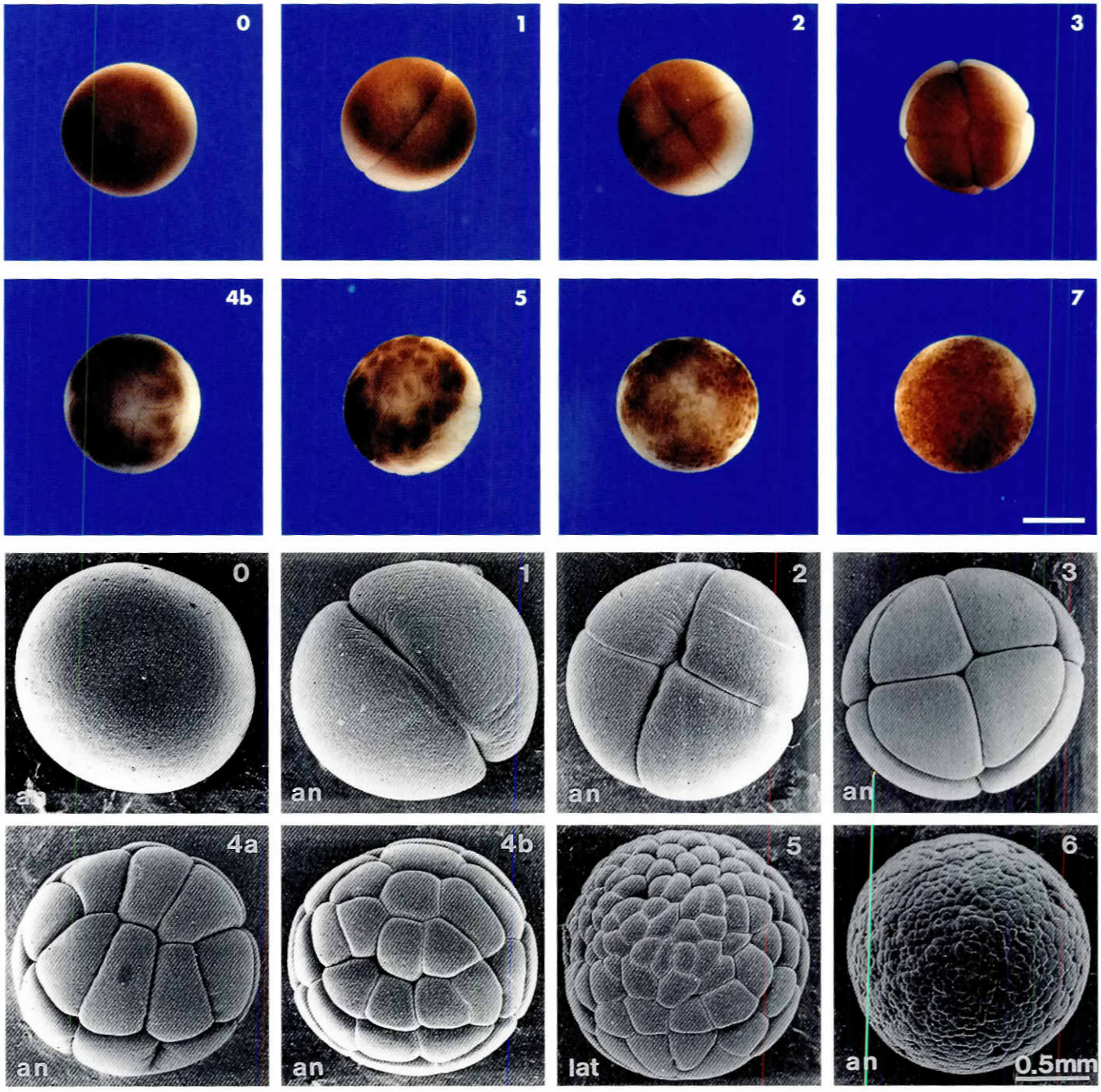

Fig. 2. Optical and SEM micrographs illustrating the external morphology of *Pleurodeles* embryos at different cleavage stages. *an, animal pole view; lat, lateral view. Bar in color photographs 0.7 mm.*

Gastrula stages

Stage 8a. Early gastrula; 31 h; 1.8 mm. Beginning of invagination, formation of blastopore on dorsal side. Internally bottle cells form at blastoporal groove; basal constriction of these cells results in the formation of numerous microvilli. Mesodermal cell migration not yet started.

Stage 8b. Slightly advanced early gastrula; 35 h; 1.8 mm. Blastopore extends laterally and deepens slightly. Initiation of mesodermal cell migration on dorsal blastocoel roof. Thinning of the blastocoel roof in the animal pole region by intercalation.

Stage 9. Advanced early gastrula; 38 h; 1.8 mm. Crescent-shaped blastopore, invagination extends laterally. Mesodermal cell migration is well under way. Formation of archenteron cavity on dorsal side.

Stage 10. Middle gastrula; 41 h; 1.8 mm. Horseshoe-shaped blastopore. Archenteron well advanced. Mesodermal mantle forms archenteron roof on dorsal side. Invagination of outermost cells over dorsal blastoporal lip as a "rolling in" process.

Stage 11. Large yolk plug; 46 h; 1.8 mm. Formation of circular blastopore; yolk plug represents one-third of embryo diameter. Internally, front of archenteron reaches original animal pole. Blastocoel reduced to small cavity. Initiation of invagination on ventral side.

Stage 12. Late gastrula; 51 h; 1.8 mm. Small yolk plug. Archenteron cavity extends ventrally and blastocoel cavity has disappeared. Further invagination in ventral region. Endodermal mass pushed to a "mesoderm-free" region.

Stage 13. End of gastrulation; 56 h; 1.8 mm. Blastopore a small slit and yolk plug has disappeared. Row of pigmentation on the dorsal side indicates the presence of neural folds. Archenteron enlarged from the antero-posterior axis. Invagination in ventral region more advanced, and mesoderm-free region reduced. Dorsal archenteron roof still consists of prechordal mesodermal cells.

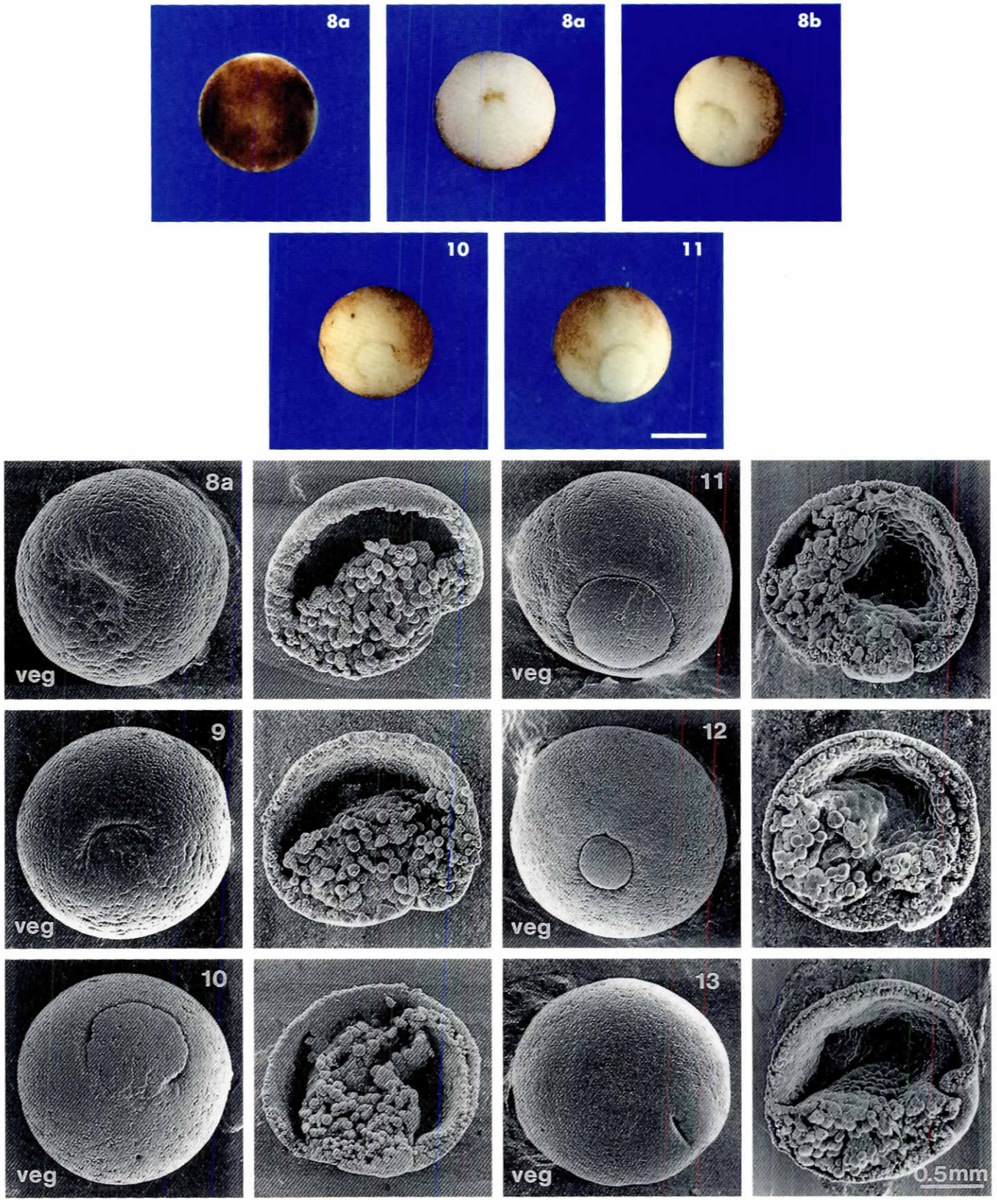

Fig. 3. Optical and SEM micrographs illustrating both external and internal morphologies of *Pleurodeles* embryos at different gastrula stages. *veg, vegetal view. Bar in color photographs 0.8 mm .*

Neurula stages

Stage 14. Initial neurula stage; 63 h; 1.8 mm. Formation of neural plate, slight elevation of neural folds. Median groove is visible on dorsal midline of neural plate. Initial individualization of notochord from 4 to 5 cells wide. Flattened endodermal cells extend dorsally to cover archenteron roof.

Stage 15. Neural plate stage; 69 h; 2.0 mm. Significant elevation of neural folds except in posterior region. Anterior neural plate bends downwards. Appearance of ciliated cells on flank of the embryo. Neural plate consists of one layer of columnar cells. Notochord further condensed. Flattened endodermal cells coming closer together dorsally underneath the notochord except in posterior region.

Stage 16. Early neural fold stage; 69.5 h; 2.0 mm. Extension of trunk neural folds toward dorsal midline. Elevation of posterior neural folds.

Stage 17. Middle neural fold stage; 70 h; 2.0 mm. Neural folds closer together in trunk region. Elevation of anterior and posterior neural folds.

Stage 18. Late neural fold stage; 71 h; 2.0 mm. Neural folds almost touch each other anteriorly and posteriorly.

Stage 19. Joining of neural folds; 74 h; 2.3 mm. Neural folds touch each other from anterior to posterior regions, suture still visible. Ciliary movement apparent. Three pairs of somites visible externally.

Stage 20. Complete fusion of neural folds; 77 h; 2.5 mm. First indication of head region, beginning of eye protrusion. Five somites visible.

Stage 21. End of neurulation; 80 h; 2.6 mm. Neural tube formed. Modulation of head which bends ventrally. Distinct eye protrusion. First indication of pronephros anlage.

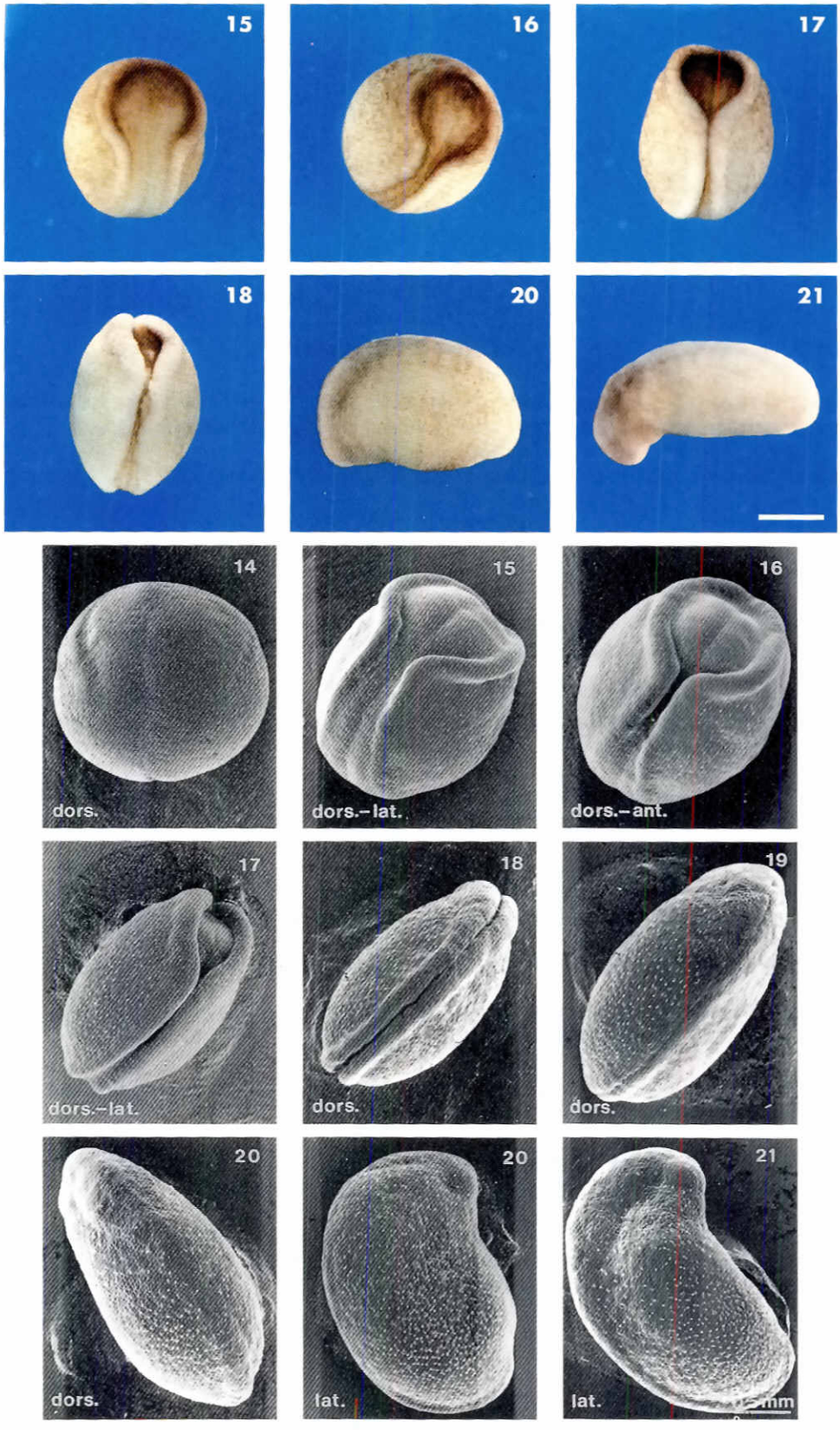

Fig. 4. Optical and SEM micrographs illustrating the external morphology of *Pleurodeles* embryos at different neurula stages. *dors, dorsal view; lat, lateral view; dors-lat, dorso-lateral view. Bar in color photographs 0.83 mm.*

Tail-bud stages

Stage 22. Initial tail-bud stage; 3 d, 15 h; 3.1 mm. Optic placode clearly visible externally. Head flexure approximately 110°. Protrusion of pronephros anlage. First appearance of gill anlage.

Stage 23. Head extension; 2 d, 19 h; 3.4 mm. Head flexure approximately 125°. First appearance of tail-bud.

Stage 24. Further head extension; 4 d; 3.8 mm. Head flexure approximately 140°. Distinct protrusion of pronephros and eye anlagen. Stomodeal region visible as a less pigmented spot. Formation of tail-bud.

Stage 25. Elongation of tail-bud; 4 d, 6 h; 4.1 mm. Convexity of head with respect to trunk less pronounced, head flexure approximately 160°. Length of tail-bud represents 1/12 of the embryo.

Stage 26. Olfactory placode visible externally; 4 d, 14 h; 4.4 mm. External gills develop as outwardly directed pockets. Protrusion of auditory vesicle in front of gill pockets. Tail-bud clearly distinct. Response of muscle to mechanical stimulus.

Stage 27. Initial formation of balancers; 5 d; 4.9 mm. Head flexure has disappeared. Dorsal surface of trunk slightly concave. Appearance of folds on gill pockets corresponding to anlage of the three pairs of gills.

Stage 28. Cone-shaped balancers; 5 d, 10 h; 5.4 mm. Formation of dorsal fin. Length of tail represents about 1/9 of embryo. Appearance of several melanophores along the somites. Beating of heart visible.

Stage 29. Individualization of first 2 gills; 5 d, 20 h; 5.9 mm. Similar morphology to above.

Stage 30. Protrusion of gills; 6 d, 6 h; 6.2 mm. Appearance of melanophores in head region. Length of tail represents 1/6 of embryo. Spontaneous muscle movement.

Stage 31. Blood circulation in first 2 gills; 7 d; 7.1 mm. Opaque cylindrical balancers. Appearance of blood circulation in anterior region of tail.

Stage 32. Blood circulation established in all 3 pairs of gills; 8 d; 8.1 mm. Cylindrical gills. Trunk melanophores oriented as longitudinal and transverse bands along somites.

Stage 33. Branching of gills; 9 d; 10.4 mm. Formation of anterior limb bud. Translucent balancers. Numerous melanophores in head and on somites. Extension of blood circulation to 2/3 of tail. Length of tail represents 1/3 of embryo. Cornea becomes transparent.

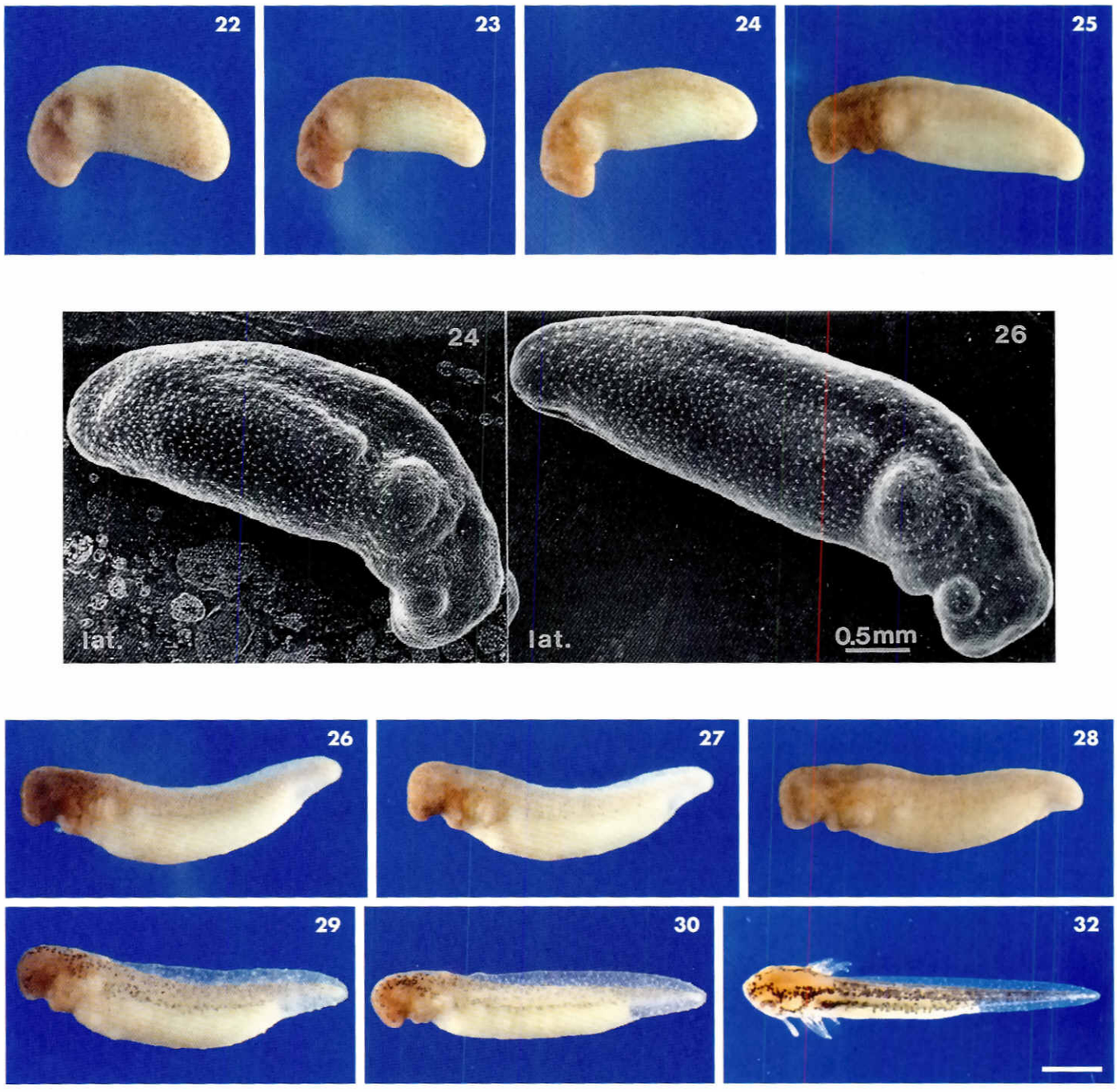

Fig. 5. Optical and SEM micrographs illustrating the external morphology of *Pleurodeles* embryos at tail-bud stages. *lat, lateral view. Bar in color photographs: from stage 22 to 25, 0.91 mm; from stage 26 to 28, 1.02 mm; from stage 29 to 30, 1.06 mm; stage 32, 1.75 mm.*

Larval stages

Stage 34. Initial hatching stage; 11 d; 11.1 mm. Cone-shaped anterior limb bud. 4 to 6 branchings in each gill. Elongation of balancers.

Stage 35. Middle hatching stage; 12 d; 11.3 mm. Cylindrical anterior limb bud. Thinning of distal end of balancers. Internally, beginning of mesenchymal condensation in future head cartilage and in limb buds.

Stage 36. Late hatching stage; 13 d; 11.5 mm. Larva transparent, internal organs including heart, stomach, visceral arches and auditory capsules visible. Regression of balancers.

Stage 37 (previously stages 37 and 38). Opening of mouth; 17 d; 12 mm. Beginning of feeding. Anterior limb bud developed as a paddle. Several melanophores on dorsal fin and around cloaca. Resorption of yolk. Extension of blood circulation to entire tail.

Stage 38 (previously stages 39 and 40). Two digits formed in anterior limb; 20 d; 13 mm. Yolk entirely resorbed. Melanophores scattered in gills and in tail. Elongation of gills and augmentation of branchings. Chondrification of Meckel's cartilage and of other visceral arch cartilage. Balancers reduced.

Stage 39 (previously stages 41 and 42). Formation of 3 digits in forelimb; 28 d; 16 mm. Numerous melanophores in dorsal fin. Initial formation of posterior limb bud.

Stage 40 (previously stages 43 and 44). Initial formation of forth digit in forelimb; 36 d; 17 mm. Elongation of first 2 digits in forelimb. Cone-shaped posterior limb bud.

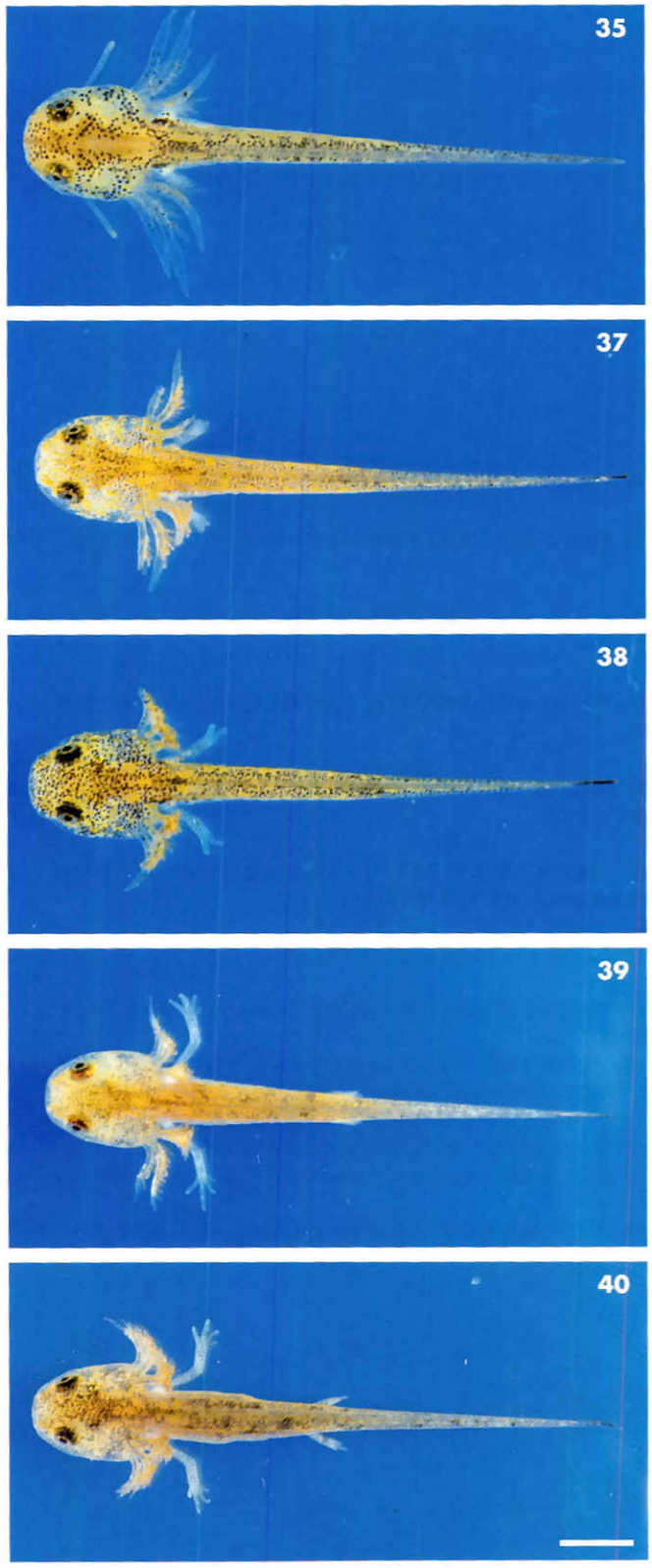

Fig. 7. *Pleurodeles* larvae at stages 35 to 40. *dor, dorsal view. Bar: stage 35, 1.35 mm; stage 37, 1.48 mm; stage 38, 1.55 mm; stage 39, 1.95 mm; stage 40, 2.2 mm.*

Larval stages (cont.)

Stage 41 (previously stages 45 and 46). Forth digit of forelimb formed; 43 d; 18 mm. Cylindrical posterior limb bud elongated.

Stage 42 (previously stages 47 and 48). Paddle-shaped posterior limb bud with V-shaped cut in middle; 50 d; 19 mm.

Stage 43 (previously stages 49 and 50). Two digits formed in hindlimb; 57 d; 23 mm. Initial formation of third digit of hindlimb.

Stage 44 (previously stages 51 and 52a). Formation of third digit in hindlimb; 64 d; 25 mm. Initial formation of forth digit in hindlimb.

Stage 45 (previously stages 52b and 53). Formation of forth digit in hindlimb; 72 d; 30 mm. Elongation of all digits. Initial formation of fifth digit in hindlimb.

Stage 46 (previously stage 54). Formation of fifth digit in hindlimb; 79 d; 38 mm.

Stage 47 (previously stage 55a). External gills fully developed; 90 d; 44 mm.

Stage 48 (previously stage 55b). Gills reduced to half; 100 d; 60 mm. Fin regression. Thickening of anterior and posterior limbs. Beginning of skin transformation in trunk region.

Stage 49 (previously stage 55c). First moulting, gills reduced; 105 d; 65 mm. Head becomes flattened. Regression of tail fin, width of tail reduced to half. Beginning of respiration by lung.

Stage 50 (previously stage 56). Metamorphosis completed; 110 d; 72 mm. All fins and gills completely disappeared.

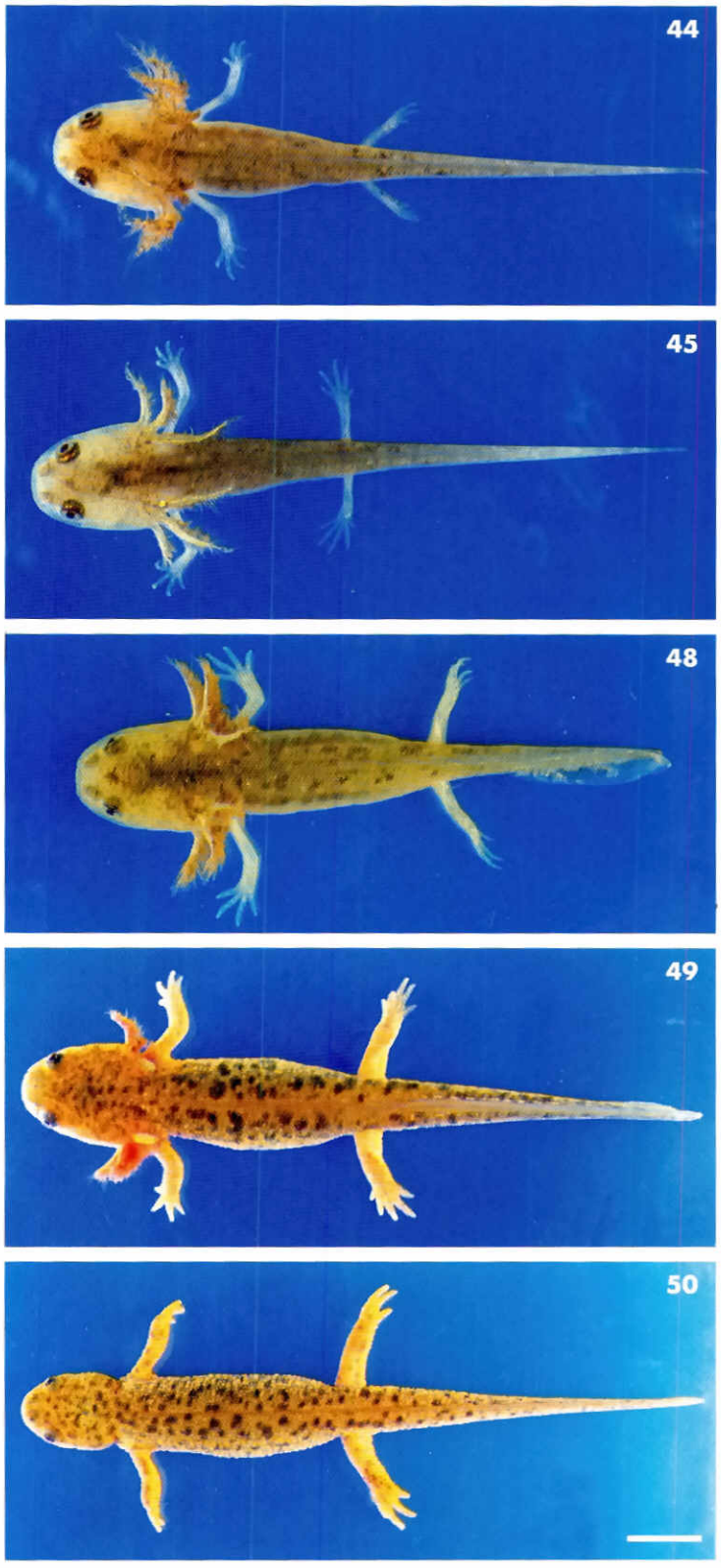

Fig. 7 (cont.). *Pleurodeles* larvae from stage 44 to the end of metamorphosis. *dor, dorsal view. Bar: stage 44, 3.023 mm; stage 45, 3.65 mm; stage 48, 7.96 mm; stage 49, 7.74 mm; stage 50, 8.5 mm.*