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Developmental Stages Until Hatching of the Lake Victoria Cichlid Haplochromis piceatus (Teleostei: Cichlidae)

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ABSTRACT Because little is known about embryonic developmental stages in any haplochromine cichlid, we describe here a series of normal stages of the Lake Victoria cichlid *Haplochromis piceatus*. We collected 273 embryos and scored them for 47 morphological characters. The result was an illustrated series of 12 stages from embryonic shield until hatching based on live, fixed, and histological material. We defined each stage according to a single "key" character that applied to all embryos of that stage. Other characters forming part of the stage descriptions were not necessarily present in all embryos of a stage. We compare our findings with published studies of other freshwater teleosts and find wide variation in staging systems. Our data will form a baseline for further research on cichlid development. J. Morphol. 270:519–535, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: cichlid; haplochromine; Lake Victoria; embryo; evolution; development; stages

INTRODUCTION

The haplochromine cichlids of Lake Victoria (East Africa) have been intensively studied in the last few decades, generating much information about their speciation (Greenwood, 1974; Meyer et al., 1990; Seehausen et al., 1997a; Galis and Metz, 1998; Nagl et al., 2000; Verheyen et al., 2003; Maan et al., 2004; Watanabe et al., 2004; Seehausen, 2006; Verzijden and ten Cate, 2007), as well as how they were affected by the introduction of the Nile perch and the environmental degradation in and around the lake (Barel et al., 1985; Ogutu-Ohwayo, 1990; Witte et al., 1992, 2008; Seehausen et al., 1997b; Balirwa et al., 2003).

In view of the scientific interest in Lake Victoria haplochromine cichlids, it is surprising that relatively little is known about their embryology. Fujimura and Okada (2007) discuss several studies that describe the developmental sequence of various cichlids, but there is almost no research done on the development of the haplochromine cichlids of the Lake Victoria basin, which apart from Lake Victoria comprises lakes Edward, George and Kyoga (Greenwood, 1980; Verheyen et al., 2003). Otten (1982) described the development of *Haplochromis* [*Astatotilapia*] *elegans* from Lake George, Uganda, but he focused on the period after hatching. For the description of the early embryonic stages, he refers to the developmental stages described by Jones (1974). It is noteworthy that Jones made a description of the development of substrate-brooding cichlids, whereas Otten (1982) described the development of haplochromines, which are female mouth brooders. Furthermore, there are a dissertation on ontogeny of the head part of *H. elegans* (Ismail, 1979) and a report on the development of the pharyngeal jaw muscles of *H. elegans* (Aerts, 1980).

A normal description of the ontogeny of the haplochromine cichlids is important because conservation efforts may require information about fish reproduction, and because evolutionary studies increasingly take account of developmental mechanisms in the embryo. In Lake Victoria, several environmental changes have taken places during the last few decades that may affect cichlid embryos. Two important changes are as follows: i) the decrease in dissolved oxygen levels (Hecky et al., 1994; Wanink et al., 2001) and ii) an increase of heavy metal concentrations in the lake (Kishe and Machiwa, 2003). Both can interfere with the development of fish embryos (Yediler and Jacobs, 1995; Weis and Weis, 1995a,b; Latif et al., 2001).

Pilot studies in our laboratory on *Haplochromis* [*Astatotilapia*] *piceatus*, *H.* [*Labrochromis*] *ishmaeli*, *H. thereuterion*, and *Astatoreochromis alluaudi* showed that there are differences between embryos developing under normoxic conditions and under hypoxic conditions (J. 't.Hoen, E. Burgerhout, and F. Witte, unpublished data). It was found that the head is narrower in the hypoxia group than in the normoxia group, at the moment of erythrocyte development and hatching. The hypoxia group also hatches at a later time. Therefore, there is at least some potential for

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phenotypic plasticity during development. This makes the Lake Victoria cichlids of high interest in the new field of ecological developmental biology, which aims to link environment and phenotype via development (Sultan, 2007).

A good example of this approach is the study of Fishelson (1966) in which the developmental variation between mouth-brooding and substrate-breeding was compared in three tilapiine species. It was found that substrate brooders had relatively accelerated development of certain sensory, motor, and vascular characters, and that these heterochronies (developmental timing shifts; Richardson, 1995; Klingenberg, 1998) could be linked to the difference in the environment of the early embryos. Qualitative differences were also seen, such as the presence of a fully developed cement organ in embryos of the substrate brooder(s), whereas only a rudimentary homologue was found in embryos that develop within the mother's mouth.

During the last few decades, the populations of several Lake Victoria cichlid species have strongly declined after the Nile perch boom (Witte et al., 1992; Goudswaard et al., 2008) and recovered again when Nile perch declined due to heavy fishing (Seehausen et al., 1997b; Witte et al., 2000, 2007). There is evidence in some "recovered" species of potentially adaptive changes in adult morphology of the species concerned (Witte et al., 2008). It will be interesting to determine whether the recovery process involved any changes in developmental patterns that could explain such "new" morphologies.

This study involves the cichlid *Haplochromis piceatus* (see Fig. 1), which is endemic to Lake Victoria. This species used to be common over muddy and sandy bottoms in both exposed and sheltered areas at a depth range of 2–22 m (Greenwood and Gee, 1969; Witte and Witte-Maas, 1987). Most of the adult fish lived at a depth range of 6–11 m and most of the juveniles at a depth range of 2–7 m (Witte and Witte-Maas, 1987; Goldschmidt et al., 1990). *Haplochromis piceatus* was mainly zooplanktivorous during day time, whereas during night time it fed on *Chaoborus* larvae and pupae (Goldschmidt et al., 1990).

Like the majority of the sublittoral and deepwater haplochromine species of Lake Victoria, *Haplochromis piceatus* vanished after the Nile perch upsurge in the 1980s and has not been observed in the catches since 1987 (Witte et al., 1992, 2000). However, some individuals that might belong to the species have recently been collected (M. Kishe-Machumu, unpublished data). *Haplochromis piceatus* is now bred in several aquaria around the world, including those of Leiden University. We choose this species for our study because, among the species kept in our laboratory, it is morphologically the most "generalized" cichlid *sensu* Greenwood and Gee (1969). Because of its generalized features, Greenwood (1980) assigned this species to the genus *Astatotilapia*. This has been disputed by Witte and Witte-Maas (1987), who noticed that the species bridges the gap between Greenwood's genera *Astatotilapia* and *Yssichromis*. Among the haplochromines that are currently recovering in the lake, most belong to species with *Yssichromis* features (Witte et al., 2000); *H. piceatus* is probably a good model for these species. Finally, in our facility here at Leiden University, *H. piceatus* is one of the most successfully reproducing species, meaning that sufficient embryos could be collected for our study.

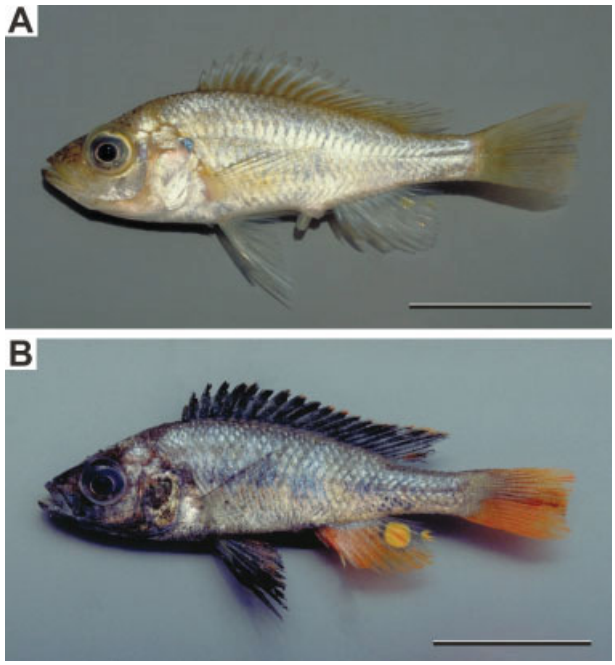

Fig. 1. Left lateral views of freshly euthanized *Haplochromis piceatus* adults. These specimens had been pinned to spread the dorsal, pectoral, and anal fins. We retouched the photos to remove the pins. No image manipulation was applied to the tissues themselves. Caught summer, 1985, Mwanza Gulf, Lake Victoria, Tanzania. (**A**) Female. Scale bar = 24.6 mm. (**B**) Male. Scale bar = 23.4 mm.

MATERIALS AND METHODS

Animal Care

Adult males and females (see Fig. 1) of *Haplochromis* [*Astatotilapia*] *piceatus* (Greenwood and Gee, 1969) were kept in aquaria of 100 × 50 × 50 cm^3 at a water-temperature of 24.1°C ± 0.5°C. The wild-type stock was collected in 1984 in the Mwanza Gulf of Lake Victoria, East Africa. They were subsequently bred for 15–16 generations in our aquaria at Leiden University. They were kept under a day–night light cycle of 12–12 h and were fed daily with commercial flake fish food (Landman, The Netherlands) alternated with a range of frozen fish foods (Aquadistri BV, The Netherlands). All procedures were carried out with approval of the local ethics committee.

Collection, Fixation and Storage of the Embryos

Haplochromis piceatus is a mouth-brooding cichlid. After fertilization, the eggs were obtained from the mouth of the females

TABLE 1. Summary of material examined and the characters for each stage

Stage	Hpf[a]	No. whole mounts[b]	No. embryos sectioned	Key character	Other characters
1	ca.12–20	10		embryonic shield	
2	ca.24	18		optic Anlagen	1–5 somites; tail Anlage; brain Anlage
3	ca.39–41	15	2	auditory placodes	3–13 somites
4	ca.45–50	39	7	tailbud: not yet lifted from the yolk sac	9–16 somites; three brain divisions: prosencephalon, mesencephalon and rhombencephalon; lens placode; olfactory placode; Anlage pharyngeal arches; heart tube; melanocytes on yolk sac
5	48–56	20		tail lift starts—tailbud separates from yolk sac	18–21 somites; optic cup; auditory vesicle; IVth brain ventricle; beating heart tube; melanocytes on yolk sac and trunk
6	66–68	21		torsion of the body axis	21–27 somites; 2–6 pair of rhombomeres; median fin fold; solid spherical lens; olfactory pit; pectoral fin: scattered cells
7	72–74	36	5	tail lift complete—tail totally separated from yolk sac	27–33 somites; heterocercal stage of the tail; cloaca; circulating erythrocytes; melanocytes over rhombencephalon
8	77–91	16	3	caudal fin Anlage	28–36 somites; operculum Anlage
9	95–104	32	7	pectoral fin a protruding bud	28–31 somites; retinal pigment; melanocytes over mesencephalon; nephric ducts ending in cloaca
10	101–110	29	6	start head straightening—upper lip visible	28–33 somites; blood vessel arcades in caudal fin
11	ca.120	7		head straightening—loss connection of lower jaw with yolk sac	29–31 somites; 2–3 hypural Anlagen; anal fin Anlage; hatching; gill filaments
12	132–150	13	1	head completely straightened	29–31 somites; mass hatching; dorsal fin Anlage; 1 parhypural; 4 hypurals; 6–10 caudal fin rays

[a]Hpf = hours post fertilization. Note that the hours post fertilization are only an indication since characters are not bound to time. That is why we use a key character method.
[b]Total number of embryos used to describe the development of *Haplochromis piceatus* is 256. Seventeen living embryos were followed to describe the development.

by manual compression. The eggs were kept in an open plastic box, with a floor of fine mesh gauze, suspended in the main aquarium. This ensured sufficient water flow and oxygen supply for the developing embryos. The oxygen saturation of the water was 8 mg/L. Unfertilized eggs and dead embryos were discarded and excluded from our analysis. At time intervals of 1 h, embryos were fixed in half-strength Karnovsky's fixative at 4°C (Karnovsky, 1965) for 4–12 h; dehydrated through a graded propan-2-ol series (20%, 40%, and 70%) and stored in 70% propan-2-ol. We used propan-2-ol in preference to ethanol, because we find that ethanol dehydration can make the yolk very hard and brittle. A total of 273 embryos ranging in age from ca. 12–150 h postfertilization were processed and subsequently analyzed.

Observations on Living Embryos

The embryos were followed during their development every 6 h until they hatched. They were kept in aquarium water at 24.1°C ± 0.5°C and observed under a stereo dissecting microscope with a maximum magnification of 63×.

Whole-Mounting of Embryos

All embryos were prepared as hematoxylin-stained wholemounts because this made the surface features much easier to discriminate. The chorion (egg envelope) was removed, the embryos washed in de-ionized water (3×), stained with Mayer's hematoxylin (30 s), and rinsed with tap-water (3×). They were then stored in 70% propan-2-ol. The wholemounts were observed with a stereo dissecting microscope (63×) and drawn with the aid of a *camera lucida* drawing tube.

Histology

For histological studies, we used embryos that were fixed at ~5 h intervals. Before embedding the embryos in paraffin, their yolk was removed as much as possible since it becomes very brittle during processing, which can make it difficult to section. The embryos were then dehydrated through a graded ethanol series and embedded in paraffin (Paramat, BDH, Poole) at 60°C with Histoclear (National Diagnostics, Atlanta) as the intermediate reagent, sectioned at 5 μm, and then triple-stained with Alcian blue and hematoxylin-eosin.

Staging Definition

This study describes the normal embryonic development from the stage of embryonic shield formation until hatching. We used a "key-character" approach for defining stages. A key character is one that is present in all embryos of that stage. Other characters contribute to the definition of the stages but are not necessarily present in all embryos of a given stage. The morphological characters we scored for each embryo are based on various published descriptions of embryonic development in other fish species. These include several tilapiine cichlids (Fishelson, 1966); several substrate brooding cichlids (Jones, 1974); blackchin tilapia (Shaw and Aronson, 1954); Nile tilapia (Morrison et al., 2001; Fujimura and Okada, 2007); stickleback (Swarup, 1958); platyfish (Tavolga, 1949); Atlantic killifish (Armstrong and Child, 1965); American shad (Shardo, 1995); zebrafish (Kimmel et al., 1995); and medaka (Iwamatsu, 2004).

Notes on the Stage Descriptions

For the description of each stage, we follow the rule that only newly appeared characters, or new transformations, are mentioned for each stage. The mentioning of a character in the

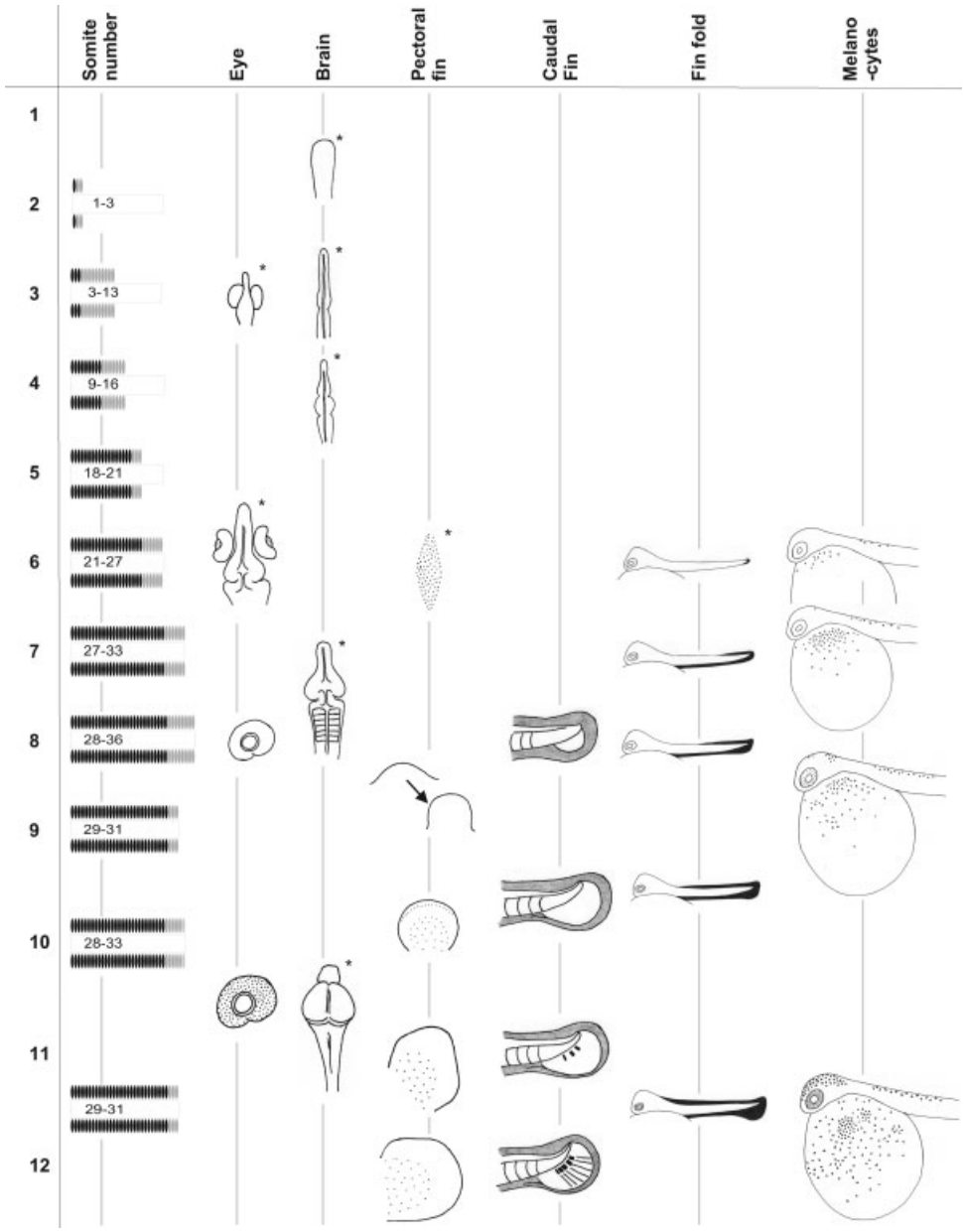

Fig. 2. Graphical overview of selected morphological characters seen at different stages of *Haplochromis piceatus* development. Characters marked with an asterisk (*) are shown in dorsal view; the others are shown in left lateral view.

figure legends, by contrast, does not necessarily indicate that it first appeared in that stage. Where a new transformation appears exceptionally in one or only a few embryos, we make a note of this in the text. To indicate the frequency of appearance of a character in a stage, we use the notation (x/y) where x is the number embryos showing that feature, and y is the total number of embryos at that stage.

RESULTS

We have studied the development of a normal series of 273 embryos (256 fixed and 17 living ones) of *Haplochromis piceatus* ranging from the start of organogenesis (appearance of the embryonic shield) until hatching. This period runs from ca. 12 to 150 hours postfertilization (hpf). A summary of our findings on the development of *H. piceatus* is given in Table 1. Graphical representations of selected characters, and their distribution over stages, are shown in Figure 2. The 12 stages are illustrated in Figure 3. Note that somite counts refer to the number of postotic somite pairs.

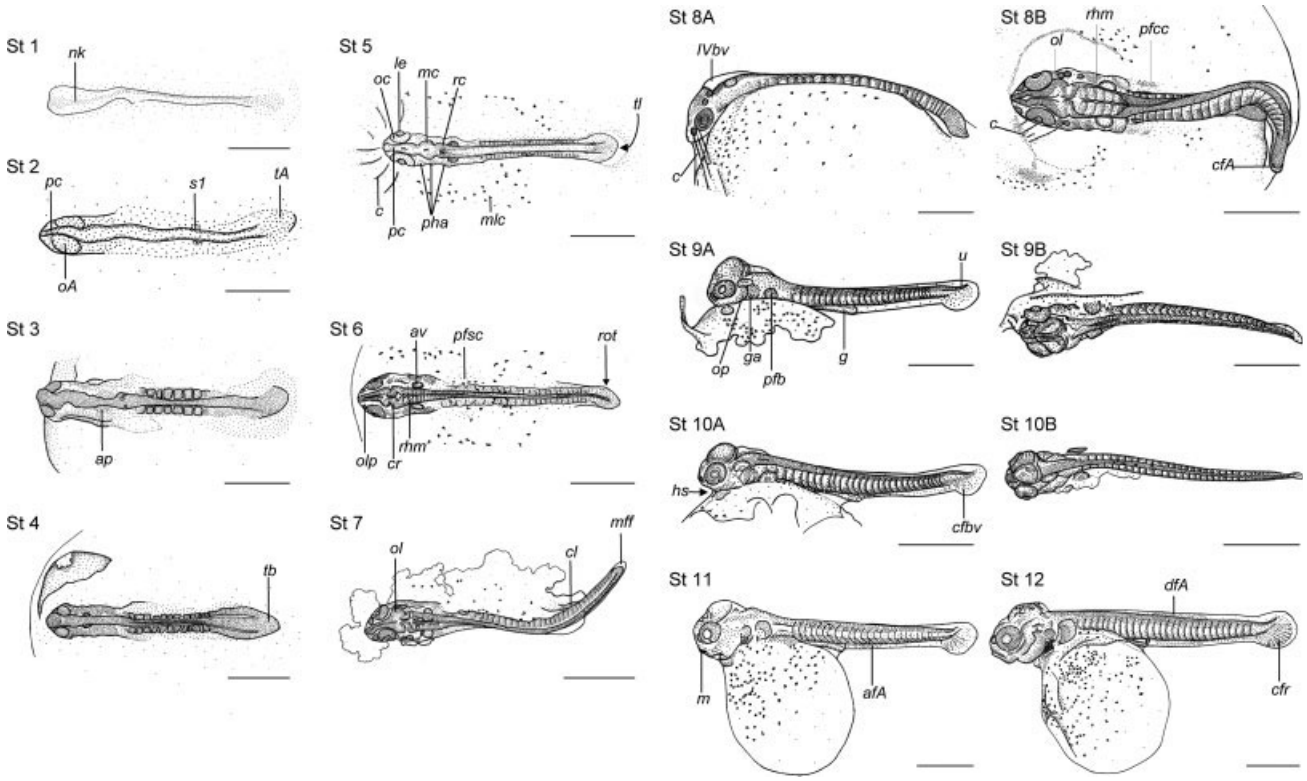

Fig. 3. Stages (St) 1–12 inclusive for *Haplochromis piceatus* embryos. Stage 1–7 are dorsal aspects; 8B, 9B, and 10B are (latero-) dorsal aspects; the remaining are left lateral aspects. In all cases, rostral is to the left of the figure. **Stage 1**: Embryonic shield. **Stage 2**: Optic Anlage. **Stage 3**: Auditory placode. **Stage 4**: Tailbud. **Stage 5**. Tail lift starts. **Stage 6**: Rotation of the body axis. **Stage 7**: Tail lift complete. **Stage 8**: Caudal fin Anlage. **Stage 9**: Pectoral fin bud. **Stage 10**: Head straightening—upper lip visible. **Stage 11**: Head straightening - loss of connection of lower jaw with yolk sac. **Stage 12**: Head straightening completed. Scale bars: stage 1–8 = 500 μm; stage 9–12 = 1 mm. IVbv, fourth brain ventricle; afA, anal fin Anlage; ap, auditory placode; av, auditory vesicles; (c), creases in membrane; cfA, caudal fin Anlage; cfbv, caudal fin blood vessels; cfr, caudal fin rays; cl, cloaca; cr, cerebellar ridge; dfA, dorsal fin Anlage; g, gut; ga, gill arches.; hs, head straightening; le, lens; m, mouth; mc, mesencephalon; mff, median fin fold; mlc, melanocytes; nk, neural keel; oA, optic Anlagen; oc, optic cup; ol, optic lobe; op, operculum; olp, olfactory pit; pc, prosencephalon; pfb, pectoral fin bud; pfsc, pectoral fin scattered cells; pfcc, pectoral fin cell condensation; pha, pharyngeal arches; rc, rhombencephalon; rhm, rhombomeres; s1, first somite; tA, tail Anlage; tb, tail bud; tl, tail lift; u, urostyl.

Stage 1: Embryonic Shield (ca. 12–20 hpf)

The embryonic shield is visible, first as a small disc and then elongating along the rostrocaudal axis (see Fig. 3). The neural keel then appears as a thickening in the ectoderm in the midline of the embryonic shield.

Stage 2: Optic Anlage (ca. 24 hpf)

The cranial part of the neural tube thickens, forming the brain Anlage which at this stage lacks a neurocoel (see Fig. 3). The optic Anlage is visible as an outgrowth of the prosencephalon. The first somites appear (1–5 pairs). The tail region is indicated as a flat, plate-like area attached completely to the yolk, but is not yet bud-like.

Stage 3: Auditory Placode (39–41 hpf)

The auditory placode is visible as an ectodermal thickening in wholemounts (see Fig. 3) and on histological sections (Fig. 4A). There is no sign yet of the lens or olfactory placodes. Three to 13 postotic somites are present. On histological sections, it is seen that the neural Anlage in the head region is laterally flattened (Fig. 4A), whereas in the caudal end of the trunk it is dorso-ventrally flattened. The trunk region shows in the midline of the body the neural keel, the notochord and the endoderm that will from the gut (Fig. 4B).

Stage 4: Tailbud (45–50 hpf)

The tail is transformed from a flat plate into a bud-like structure (see Fig. 3), not yet lifted from the yolk. In this stage, the olfactory (12/39 embryos) and lens placodes (11/39) are seen for the first time (data not shown). Three primary brain regions are present (prosencephalon, mesencephalon, and rhombencephalon) in 10/39 embryos. Anlagen of the pharyngeal arches start to appear as three thickenings in the pharyngeal wall (8/39).

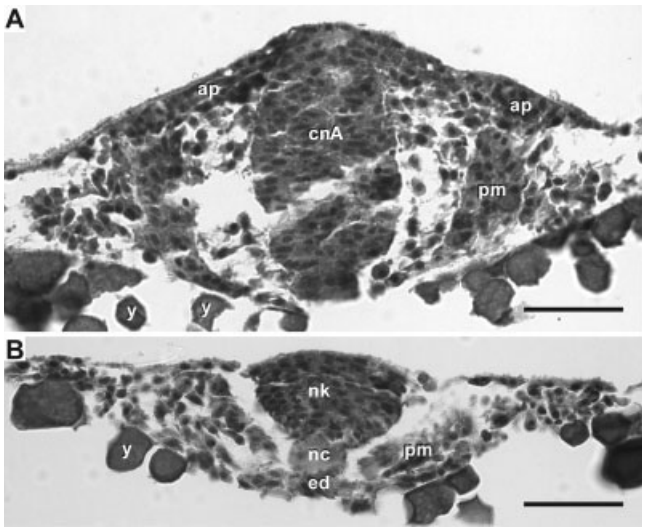

Fig. 4. Transverse sections of the head region (**A**) and the trunk region (**B**) of a stage 3 *Haplochromis piceatus* embryo. Scale bar = 50 μm. ap, auditory placode; cnA, cranial neural Anlage; ed, endoderm; nc, notochord; nk, neural keel; pm, paraxial mesoderm; y, yolk.

Cranial to the head the heart tube is seen in 3/39 embryos. In one embryo, melanocytes appear on the surface of the yolk sac. In another embryo, there is already sign of a developing rhombomere pair which in most embryos appear during stage 6.

Stage 5: Tail Lift Starts (48–56 hpf)

In stage 5, the tip of the tailbud starts to separate from the yolk, a process that spreads craniad, lifting the tail out of the yolk groove (see Fig. 3). This separation corresponds to the "tail lift" of Shardo (1995). In the developing brain, the wall of the mesencephalon forms a thickening that will become the optic lobes in stage 6. The fourth brain ventricle is visible in 4/20 embryos. The auditory placode transforms into a vesicle (3/20 embryos).The heart tube beats irregularly (10/20). Three to nine melanocytes can be found on the trunk (at the axial level of somites 4 to 11 of the embryo). In some cases, after removing the yolk sac, the first melanocytes are visible in the gut wall. There are 18–21 somites.

Stage 6: Rotation of Body Axis (66–69 hpf)

The tail is lifted from the yolk sac to the axial level of somites 19–21. At the same time, the embryo starts to undergo rotation (torsion) around the primary axis (see Fig. 3). As a result, either the left or right aspect of the tail, and later also of the trunk, comes to lie against the yolk. Torsion starts from the caudal end of the embryo at this stage and progresses rostrad in later stages. *Note*: this is in contrast to the chicken embryo, in which torsion begins at the head end (see Hamburger and Hamilton, 1951). Torsion ceases when the caudal end of the head-region is reached. Sporadic, spontaneous muscular contractions of the body begin at this stage, and the embryo can flip its body so that it lies alternately with its left and then right side against the yolk. In the developing brain the optic lobes, cerebellar ridge and rhombomeres (a total of six pairs at the end of this stage) are formed. The fourth ventricle is visible in all embryos. The epiphysis can be seen in the diencephalic roof. The lens is a solid, spherical cell mass of cells situated within the optic cup and is still attached to the surface ectoderm. On the ventral aspect of the optic cup, the choroid fissure is visible. The olfactory placode starts to invaginate and is visible as a pale spot on the surface. The heart tube starts looping at the end of this stage. The median fin fold is growing around the tip of the tailbud (6/21 embryos). A total of 21–27 somites are present. Furthermore, scattered cells are seen forming the Anlage of the pectoral fin (a cell condensation; 12/21) lateral to the embryo on the yolk sac, at a level of the first to third somite.

Stage 7: Tail Lift Completed (72–74 hpf)

The tail lift is completed so that the tail is entirely separated from the yolk sac (see Fig. 3). The ventral side of the trunk of the embryo stays connected to the yolk sac, while the embryo itself rotates. The torsion of the embryos extends to the 10th somite at the beginning of this stage and to the third somite at the end of this stage. There are 27–33 pairs and each somite has an epimeric and hypomeric region. Somites can be scored as either precloacal or postcloacal. The cloaca has become visible as a cell mass within the median fin fold. The olfactory pit is now deeply invaginated. The looping heart tube beats regularly. Erythrocytes are visible in the circulation (6/36 embryos). Two otoliths are present in the auditory vesicle. The median fin fold runs from the cloaca round the tail and up to the 10th–15th somite level dorsally. Melanocytes start to appear over the rhombencephalon. The tip of the neural tube and notochord in the tail start to bend dorsally, forming the beginning of the heterocercal stage of the tail (5/36 embryos). For a description of the remodeling of the tail tip to form the urostyle, the pointed, terminal part of the notochord, see Fishelson (1966).

The lens is separated from the overlying ectoderm (Fig. 5A). Dorsolateral to the gut nephric ducts are seen (Fig. 5F). The caudal end of the gut fuses with the body wall—forming the anus—which does not yet open to the exterior.

Stage 8: Caudal Fin Anlage (77–91 hpf)

The median fin fold, which was previously uniform in height around the tail, is now higher on

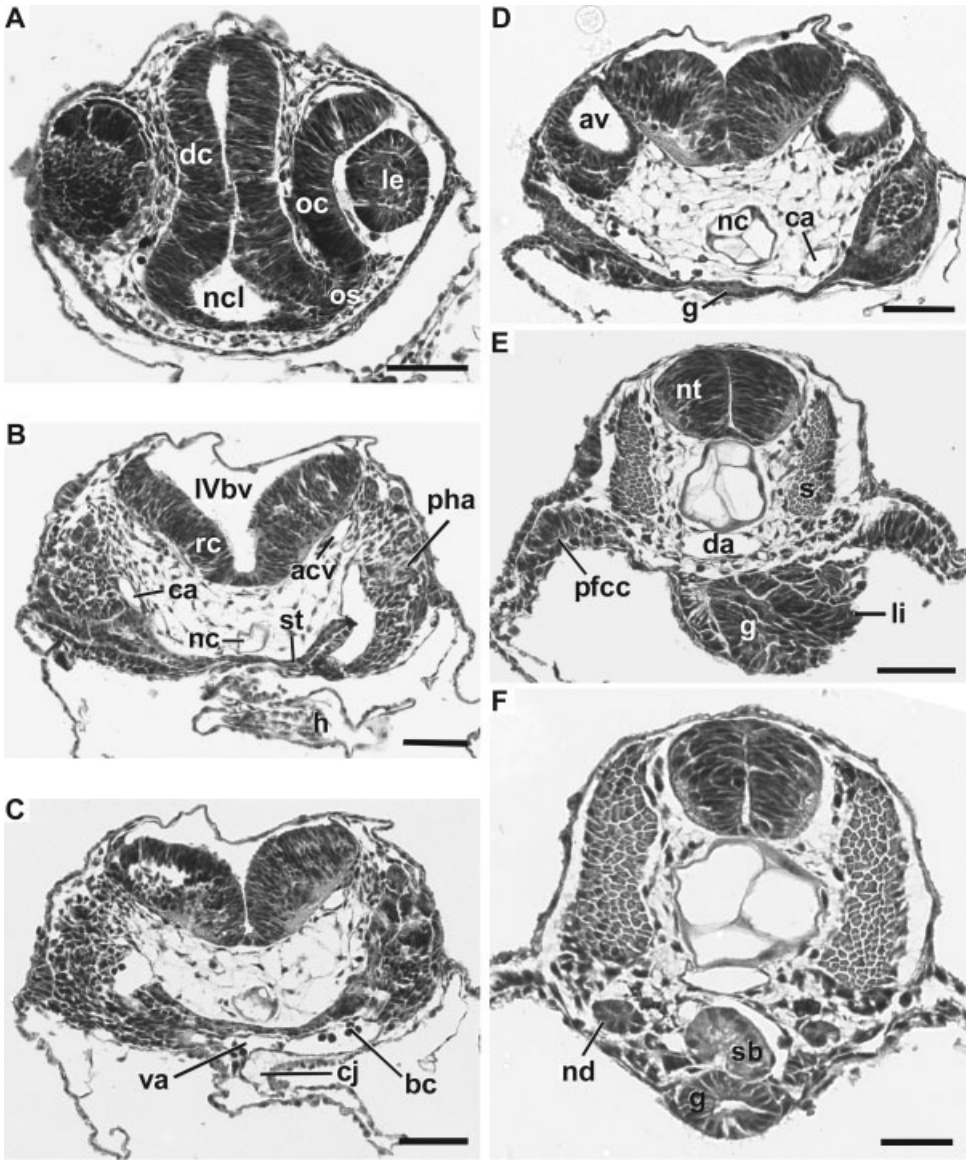

Fig. 5. Transverse sections of *Haplochromis piceatus* embryos (stage 7). (**A**) Head region. The optic cup encloses the lens and is connected to the diencephalon by the optic stalk. The lens contains lens fibers. The neurocoel is present in the cranial neural tube. (**B**) Rostral region of rhombencephalon. The neural tube has an "open book" structure forming the floor of the fourth ventricle. The pharyngeal arches do not show histological differentiation yet. The stomodeum is a broad flat structure. Carotid arteries and anterior cardinal veins are present. (**C**) Cardiac region: ventral aorta and cardiac jelly. Blood cells are present in the vascular lumen. (**D**) Caudal region of rhombencephalon. The auditory vesicle contains one compartment and lies at the lateral wall of the rhombencephalon. The prominent notochord has a number of vacuolated cells. (**E**) Pectoral region. The pectoral fin is indicated as a mesenchymal cell condensation lateral to the embryonic axis. The dorsal aorta is visible ventral to the notochord. The liver can be seen as an outgrowth of the gut. (**F**) Trunk region. The swim bladder is present as an outgrowth of the gut. The nephric ducts are seen dorsolateral to the gut. Scale bars = 500 μm. IVbv, fourth brain ventricle; acv, anterior cardinal vein; av, auditory vesicles; bc, blood cells; ca, carotid arteries; cj, cardiac jelly; da, dorsal aorta; dc, diencephalon; g, gut; h, heart; le, lens; li, liver; nd, neprhic duct; nc, notochord; ncl, neurocoel; nt, neural tube; oc, optic cup; os, optic stalk; pfcc, pectoral fin cell condensation; rc, rhombencephalon; pha, pharyngeal arches; s, somite; sb, swim bladder; st, stomodeum; va, ventral aorta.

the ventral aspect of the tail. In this region, there is a mesenchymal cell condensation indicating the onset of the caudal fin Anlage (Figs. 3 and 6A). The dorsal part of the median fin fold extends cranially to 7th–12th somite level. The operculum is growing caudad from the hyoid arch (7/16 embryos). The hyobranchial cleft is visible as a light spot. Caudal to this cleft, four branchial arches are developing. The somite count ranges from 28 to 36; however, most embryos contain 33–34 somites. The trunk is connected to the yolk sac from the first somite rostrally to the 10th–15th somites caudally. Torsion extends cranially to 5th–7th somites. Live embryos flap their tail over the

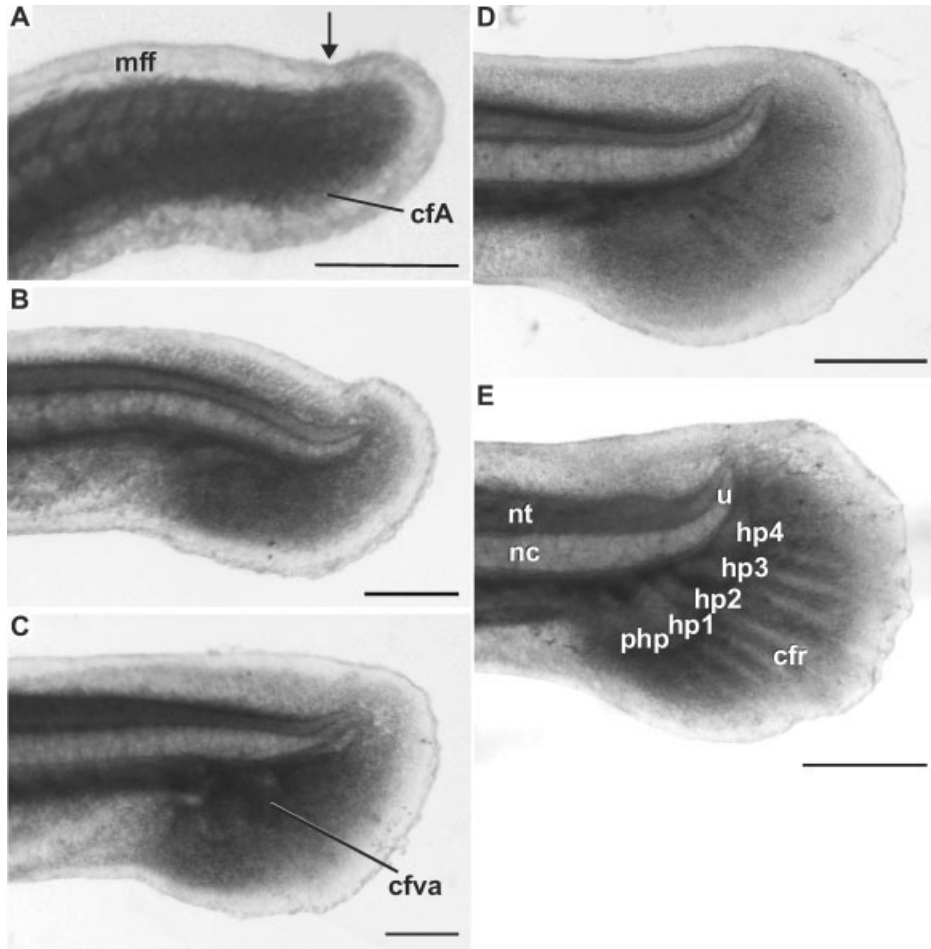

Fig. 6. Left lateral views of the tail region of *Haplochromis piceatus* embryos showing the development of the caudal fin. In all cases, dorsal is to the top of the figure and rostral to the left. (**A**) In stage 8, a mesenchymal cell condensation appears in the median fin fold ventral to the notochord. The tip of the notochord and the neural tube are bending dorsally (arrow) marking the beginning of the heterocercal stage of the tail. (**B**) Stage 9. (**C**) Stage 10, showing the developing blood vessel arcades in the caudal fin Anlage. (**D**) Stage 11. (**E**) Stage 12. Hypurals 1–4, the parhypural and fin rays are visible. Scale bars: stage 8–10 = 125 μm; stage 11–12 = 250 μm. cfA, caudal fin Anlage; cfva, caudal fin vascular arcades; cfr, caudal fin rays; mff, median fin fold; nt, neural tube, nc, notochord, php, parhypural; u, urostyle.

surface of the yolk sac. The mesenchymal cell condensation of the pectoral fin starts to form a low mound projecting slightly above the yolk sac surface; however, it is not yet bud-like.

Stage 9: Pectoral Fin Bud (95–104 hpf)

In stage 9, the pectoral fin Anlage has grown out to form a pectoral fin bud located lateral to the trunk on the yolk sac at the axial level of somites 1–3 (Figs. 3 and 7A; see histological appearance in Fig. 8E). At the tip of the pectoral fin bud, an apical ectodermal ridge (AER) develops. Blood vessel arcades develop in the caudal fin Anlage. Torsion extends to the first or fifth somite. The retina is pigmented and the lens (in fixed material) is becoming opaque. The olfactory bulb can now be seen. Rhombomeres become indistinct. Melanocytes are appearing over the optic lobes. The beating heart lies at the left side of the embryo. The operculum covers the first branchial arch. The dorsal part of the median fin fold extends to the level of 6th–13th somites. A patent gut and nephric ducts are clearly seen at this stage (Fig. 9A).

The infundibular and thyroid diverticula are visible (Fig. 8A,B,D). The two nephric ducts are fusing in the midline with each other and with the cloaca (see Fig. 10). The gut tube is completely patent (Fig. 10F,G) and the cloaca is open to the exterior.

Stage 10: Head Straightening—Upper Lip Visible (97–120 hpf)

The straightening head starts to lift off the yolk sac (Figs. 3 and 7B). At the same time, the lower jaw elongates. This process results, during this stage, in the dorsal part of the mouth (upper lip)

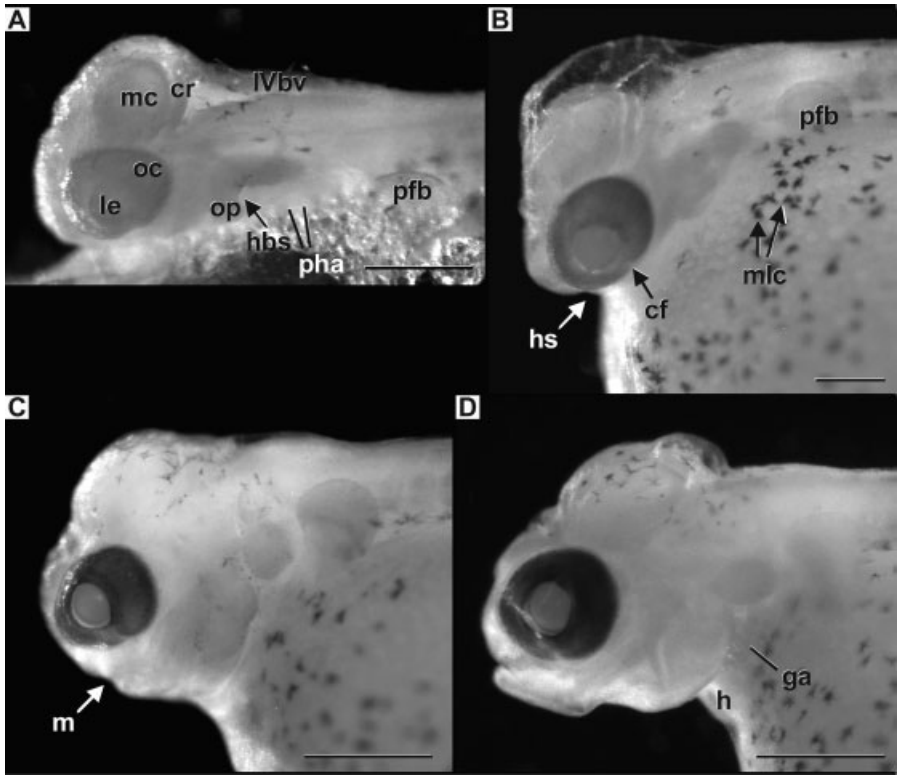

Fig. 7. Left lateral views of head region of *Haplochromis piceatus* embryos showing head straightening and elongation and the development of the pectoral fin at four different stages. In all cases, dorsal is to the top of the figure and caudal to the right. (**A**) Stage 9, showing the pectoral fin as a bud lateral to the embryo body. The apical ectodermal ridge is formed on the margin of the fin bud (here seen as a dark line). (**B**) Stage 10. Head straightening starts (arrow). (**C**) Stage 11. (**D**) Stage 12. Scale bars: stages 9 and 10 = 250 μm; stages 11 and 12 = 500 μm. IVbv, fourth brain ventricle; cf, choroid fissure; cr, cerebellar ridge; ga, gill arches; h, heart; hbs, hyobranchial slit; hs, head straightening; le, lens; m, mouth; mc, mesencephalon; mlc, melanocytes; oc, optic cup; op, operculum; pfb, pectoral fin bud; pha, pharyngeal arches.

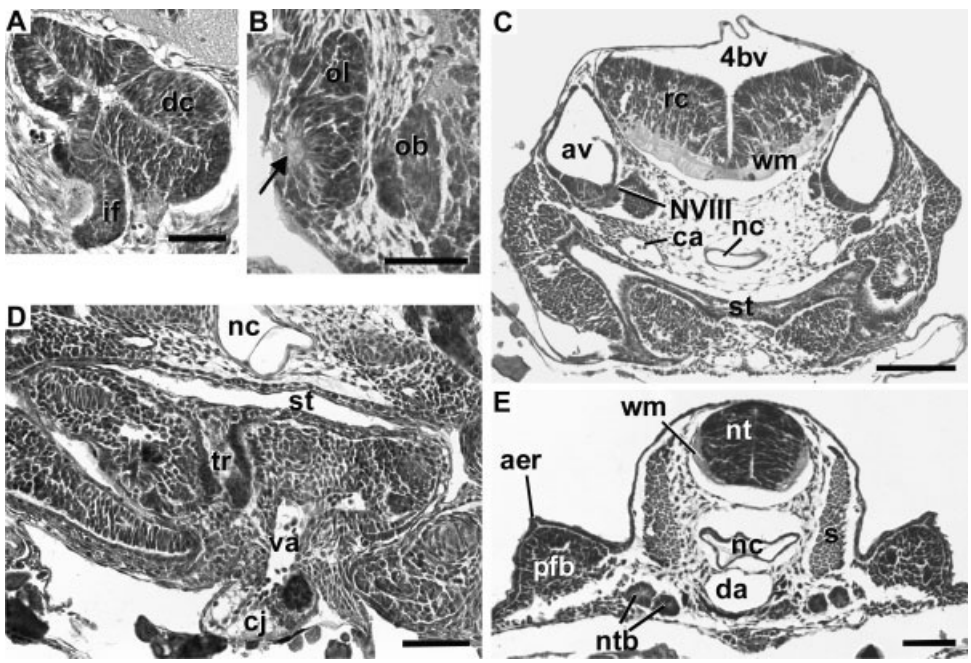

Fig. 8. Transverse sections of stage 9 *Haplochromis piceatus* embryos. (**A**) Infundibulum. (**B**) The olfactory pit is clearly invaginated (arrow; invagination starts at stage 6). The olfactory bulb of the brain is connected to the olfactory epithelium by the olfactory nerve. (**C**, **D**) Sections at the level of the rhombencephalon. In this region, the typical morphology of the gill arches is seen, containing cartilage, nerve tissue and muscles. The thyroid is present (D). (**E**) Pectoral fin buds with an apical ectodermal ridge. Scale bar = 500 μm. aer, apical ectodermal ridge; av, auditory vesicle; ca, carotid artery; cj, cardiac jelly; da, dorsal aorta; dc, diencephalon; if, infundibulum; NVIII, auditory nerve; nc, notochord; nt, neural tube; ntb, nephric tubule; ob, olfactory bulb; ol, olfactory pit; rc, rhombencephalon; s, somite; st, stomodeum; tr, thyroid; va, ventral aorta; wm, white matter.

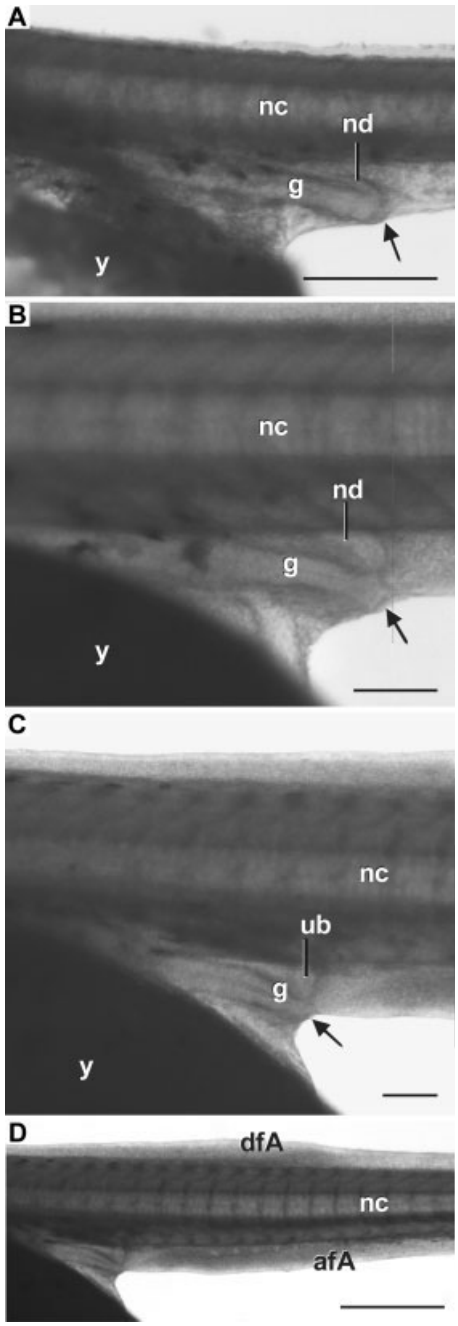

Fig. 9. Left lateral view of the mid-trunk region, including the cloaca, in *Haplochromis piceatus* embryos. Dorsal is to the top and rostral to the left. (**A**) Stage 9. The gut tube is completely patent in this stage, and the nephric ducts are fused together in the midline with one another and with the wall of the cloaca (as confirmed by histology; see Fig. 10). (**B**) Stage 11. (**C**) Stage 12. (**D**) Dorsal fin Anlage and anal fin in stage 12. Scale bar; A = 250 μm; B, C = 125 μm; D = 500 μm. Arrow, position of anus. afA, anal fin Anlage; dfA, dorsal fin Anlage; g, gut; nc, notochord; nd, nephric duct; ub,urinary bladder; y, yolk sac.

becoming exposed to the observer, whereas at earlier stages it was facing toward the yolk sac and therefore hidden. The total number of somites varies from 28 to 33; however, most of the embryos have 31 or 32 somites. The pectoral fin is displaced medially so that it now approaches the lateral aspect of the trunk. The dorsal part of median fin fold extends to 5th–11th somite level. The retina has distinct pigment and sensory layers; the latter is divided in turn into nuclear, ganglionic, and fibrous layers (data not shown). The auditory vesicle contains two compartments—the utriculus and sacculus—and is surrounded by cartilage of the otic capsule (see Fig. 11).

Stage 11: Head Straightening—Loss of Connection of Lower Jaw with Yolk Sac (120–124 hpf)

The lower jaw loses its connection with the yolk sac (Figs. 3 and 7C). The whole mouth becomes visible and is closed by an oropharyngeal membrane. Small bud-like gill filaments are appearing on branchial arches I and II. The embryos have 29–31 somites. All the embryos have a caudal fin pointing caudal-ventrally with two to three hypural Anlagen. The anal fin Anlage has developed as a mesenchymal cell condensation in the ventral part of the median fin fold, caudal to the cloaca. Some embryos hatch at this stage.

Stage 12: Head Straightening Completed (132–150 hpf)

The head is completely straightened. The freeing of the head from contact with the yolk sac now extends to the opercular region (Figs. 3 and 7D). The operculum now covers all branchial arches in most embryos. Branchial arches III–V have filaments with lamellae. The sixth branchial arch (VI) has either very small filaments without lamellae or no filaments. There are five gill slits. On the hyoid arch two to three branchiostegal rays are formed. "Respiratory" movements of the mouth and pharynx occur. The oropharyngeal membrane is broken through. Spontaneous movements of the developing pectoral fin are seen. The caudal fin contains a parhypural and four hypural Anlagen. In the caudal fin, 6–8 fin rays can be clearly seen in unstained wholemounts for the first time (Fig. 6E). They are flanked by blood vessels forming vascular arcades. The dorsal fin bud appears as a mesenchymal condensation of cells in the median fin fold with a caudal boundary at the axial level of somite 20 (2/20 embryos; Fig. 9D). The embryos contain 29–31 somites. The caudal end of the nephric ducts is dilated and sac-like, forming the urinary bladder (Fig. 9C). All embryos have hatched.

DISCUSSION

We have described here a series of normal stages in the development (from embryonic shield until hatching stages) of the Lake Victoria cichlid

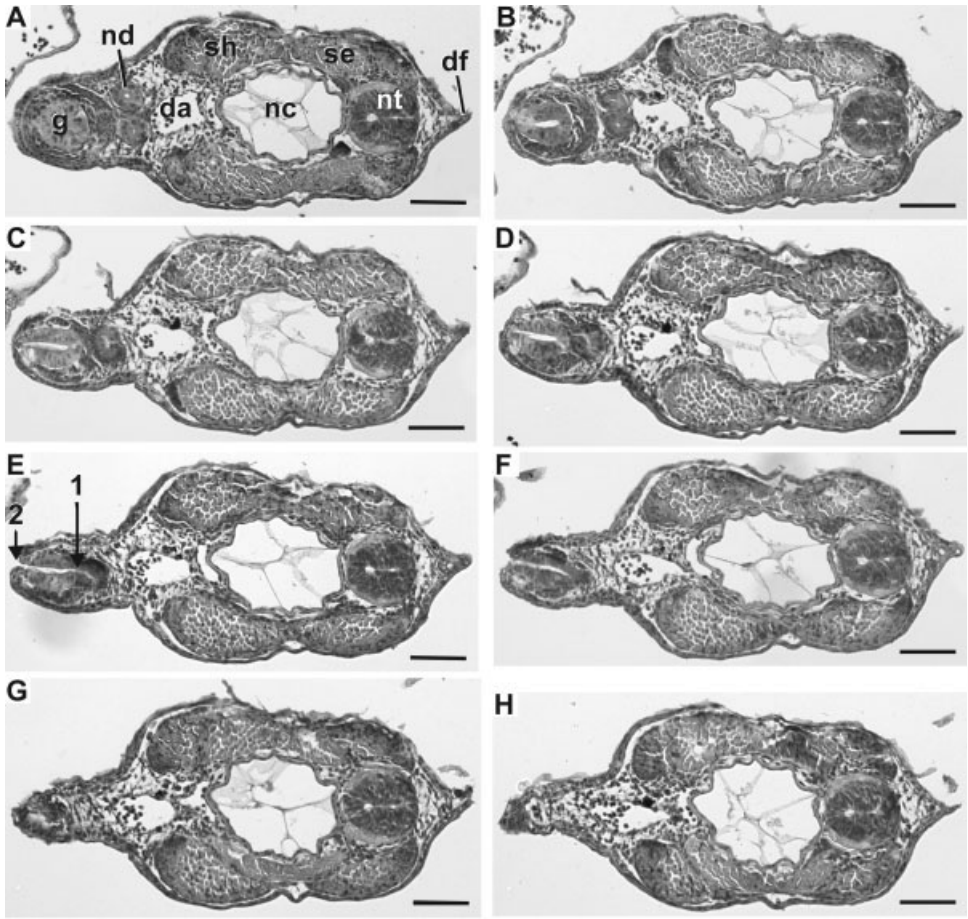

Fig. 10. (**A–J**) Series of transverse sections of a stage 9 *Haplochromis piceatus* embryo from rostral to caudal to show the morphology of the cloaca. In the most rostral section, paired nephric ducts are present (A,B); more caudally they fuse with one another (C, D) and then with the hindgut (D–F, arrow 1). During this fusion process, the hindgut itself fuses with the body wall and opens to the exterior (E, arrow 2). Scale bar = 500 μm. da, dorsal aorta; df, dorsal part of median fin fold; g, gut; nc, notochord; nd, nephric duct; nt, neural tube; se, epimeric part of the somite; sh, hypomeric part of the somite.

Haplochromis piceatus. Our primary aim was to provide a baseline for quick staging of specimens in field or lab research on the haplochromine cichlids, not to give a comprehensive description of the development of all organ systems.

We now compare our findings with those of other published studies of freshwater teleosts (for the phylogeny, see Fig. 12A). For illustrative purposes, 34 characters and their stages of appearance in different studies are given in Table 2. It can be seen that remarkably few characters are consistently present across all studies, as indicated by the number of missing data points. However, six consistently present characters have been plotted as development sequences in Figure 12B–K. From these comparisons, we can make the following remarks.

Even though the same character set is illustrated in Figure 12, there is a lack of concordance between our staging series and those of other fish species. There are several possible explanations for this lack of concordance: i) methodological issues, including observer error and stage definitions; ii) issues concerning character homology; iii) heterochrony (differences in developmental sequence or rate; reviewed by Richardson, 1995). We now discuss these points in turn.

Methodological Issues in Teleost Staging

There are many different ways of defining a staging series and, in principle, each author may have an arbitrary preference for a particular character set to define a stage. In this sense, "staging" is a personal choice. Our stages are based on a "key-character" method, as are those of Shaw and Aronson (1954) for the blackchin tilapia (*Sarotherodon melanotheron*), of Jones (1974) for substrate-breeding cichlids, of Kimmel et al., (1995) for the zebrafish (*Danio rerio*), and of Shardo (1995) for the American shad (*Alosa sapidissima*). Jones (1974) identified a common developmental

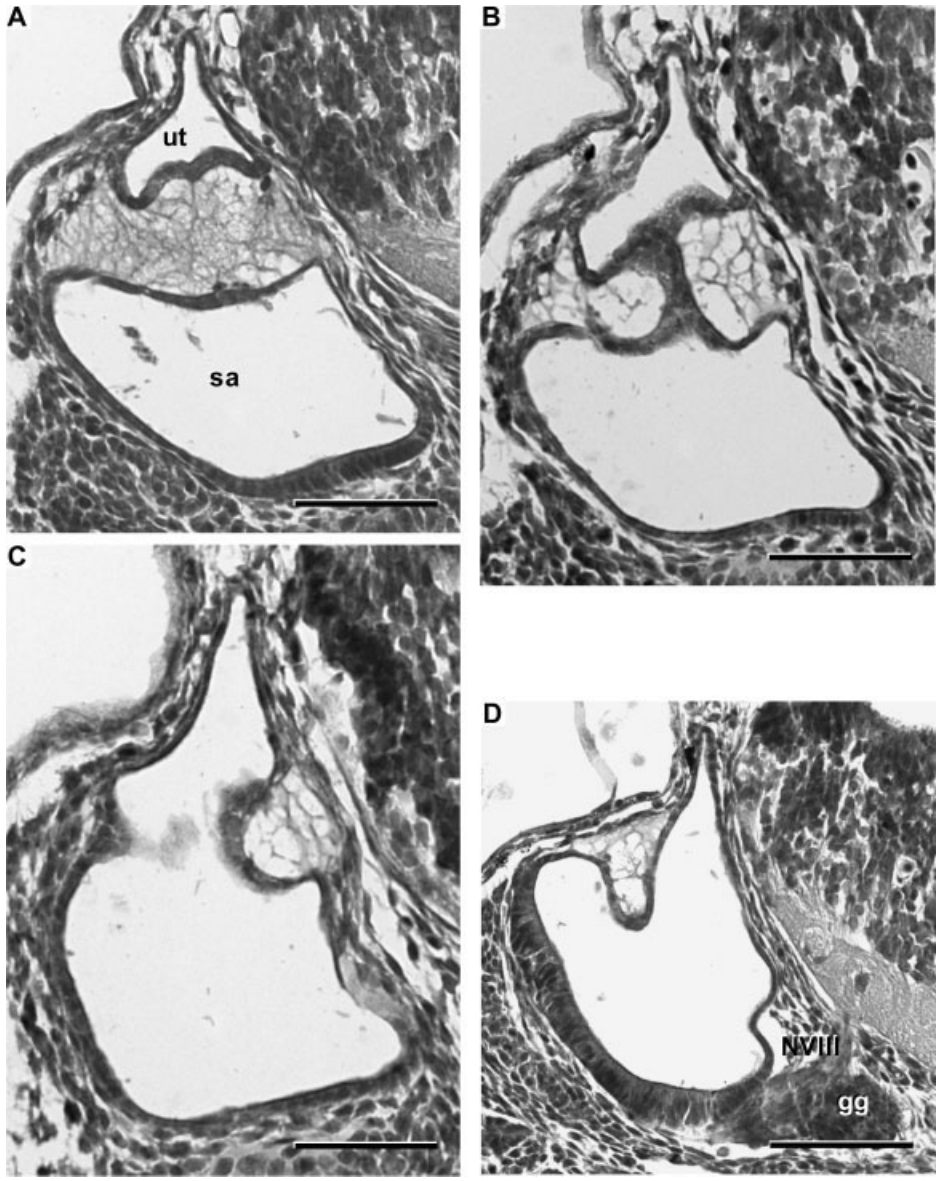

Fig. 11. Transverse section of the auditory vesicle of a stage 10 *Haplochromis piceatus* embryo. Sections from rostral (A) to caudal (D). Scale bar = 500 μm. gg, ganglion; NVIII, auditory nerve; sa, sacculus; ut, utriculus.

sequence of key characters that he could apply to several species of mouth-brooding cichlids. As he noted (Jones, 1974, p 256), the common developmental sequence of key characters that he used accommodates timing variations (heterochrony) between the species studied. This is because each

Fig. 12. Comparison of developmental sequences in 10 fresh water teleost species. (**A**) Phylogeny of the species under comparison. (**B–J**) Abacus diagrams comparing the developmental of six characters consistently present in a range of staging series. The horizontal position of the colored beads with respect to the *x*-axis indicates the first stage at which that character first develops. The series of characters on the *y*-axis is the same in each chart and is based on the sequence in *Haplochromis piceatus* (which therefore represent isochrony or euchrony). Deviation from a straight line indicates heterochrony. For example, displacement of a character to the left of its preceding character indicates acceleration or predisplacement. The characters are color-coded as follows: black, embryonic shield; yellow, optic Anlage; green, auditory vesicle; purple, heart beat; blue, pectoral fin bud; orange, retina pigment. Notes to Figure A: (1), Jones gave a single description of stages that covered all of these species in his study. He compared four "New world cichlids" with one "African cichlid." These species have subsequently been rearranged into several new genera. (2) All names are taken from fishbase.org with the exception of *Haplochromis piceatus* in which we follow van Oijen (1996). For the arrangement of the "New World cichlids" we were advised by Dr. S. O. Kullander (personal communication).

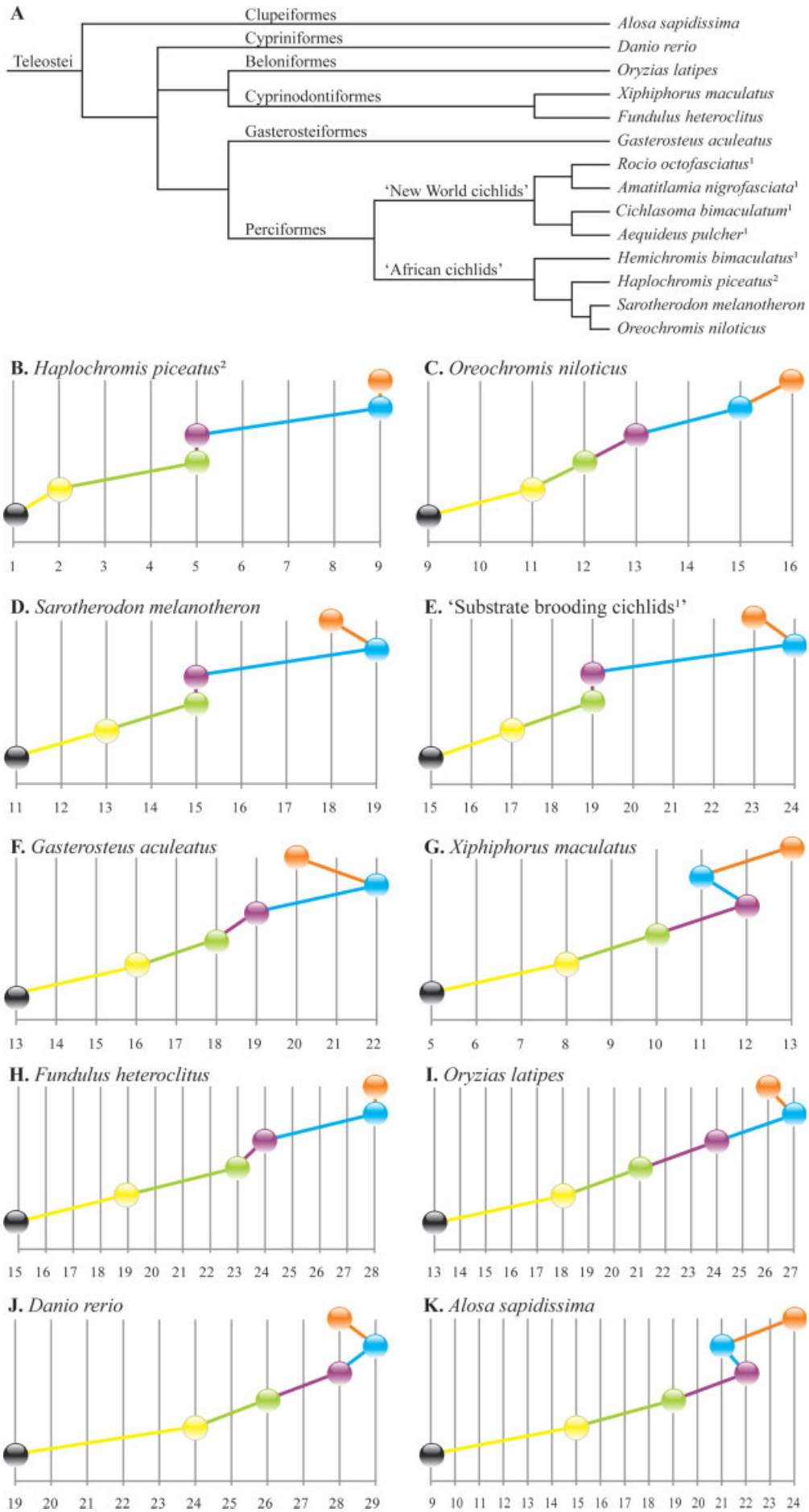

Figure 12.

TABLE 2. Selected characters and their stages of appearance in different studies

Character	This study	Shardo, 1995	Kimmel et al., 1995	Morrison et al., 2001	Fujimura and Odaka, 2007	Tavolga, 1949	Jones, 1974	Armstrong and Child, 1965	Swarup, 1958	Iwamatsu, 2004	Shaw and Aronson, 1954
Embryonic shield	1	9	19	4	9	5	15	15	13	13	11
Optic Anlage	2	15	24	5	11	8	17	19	16	18	13
Auditory vesicles	5	19	26	8	12	10	19	23	18	21	15
Heart beat	5	22	28	8	13	12	19	24	19	24	15
Pectoral fin bud	9	21	29		15	11	24	28	22	27	19
Retina pigment	9	24	28	8	16	13	23	28	20	26	18
Optic cup		18	24	7	12	10	19	22	18	21	15
First somites	2	14	23	6	10	8	15	21	17	23	12
Lens placodes	4	18	26	7	14	9	18	23		21	15
Three brain regions	4		25	7	12	10	18	20	15	20	14
Yolk sac melanocytes	4	17	30	7	11		18	23	21	22	14
Blood circulation		22	28		15	12	20	25	21	25	
Oropharyngeal membrane open	12		33	10	17		25	34	26	35	23
Caudal fin rays	12			11	18	16	25	33	28	40	24
Neural keel	1	11	23			5	16		14		11
Auditory placodes	3	17	25	6		8	18			19	13
Tailbud	4			5	11	9	19	23			15
Olfactory placodes	4	18	27	8	13	10		23			15
Rhombomeres	6	19	25	7	14	12		22			
Tail lift complete	7	21		8	14			30		26	17
Otoliths-two	7		26		13		21	26	19	25	17
Median fin fold	6	19	27		14					29	
Hatching	11				17		23	34	25	39	
Tail lift start	5	19			12			25			
Trunk melanocytes	5		28				23		21		
Cerebellar ridge	6		26	7	12	14					
Optic lobes	6				16	14		22			
Skin melanocytes over brain	7	18				15		23			21
Mesenchymal caudal fin Anlage	8					15		31			
Heterocercal stage of tail	7			10					29		19
Pectoral fin AER	9		30		15						
Vascular arcades in caudal fin	9				17			32			
Gill filaments bud-like	11		34	10							21
Heart tube starts looping	6										

The Kimmel et al. (1995) stages were not numbered in the original study; therefore for convenience, we have numbered them from 1 (one-cell stage) to 34 (protruding-mouth stage). We have also renumbered the stages of Jones (1974). Starting with his stage II,1 which we have renumbered 1 and finishing with III,12 which we have numbered as 26. We have assigned stage numbers 1 to 13 to her zygote period to early larval period (193–196 h), respectively. Blank cells, either the characters were not described or they were not clear to us. AER, apical ectodermal ridge.

of his stages was tied to only one character. Other characters (concurrent characters *sensu* Shardo, 1995) may vary in timing within and between species, relative to the key characters, without affecting the sequence of key characters itself.

In this study, we find many characters that do not segregate completely to a single stage. For example, our stage 4 is represented by 39 embryos. Among these, the olfactory and lens placodes are present in only 12 and 11 embryos, respectively. At stage 6, the median fin fold is present in six embryos but absent in the other 15 examined. At stage 7, five out of 36 embryos have begun to form a heterocercal tail, while the others have not.

If we try to define a set of stages using the same primary characters that Shardo (1995) used, we run into problems. First, we did not use the same techniques Shardo (1995) used to describe her stages of the American shad. For instance, she removed the superficial ectoderm layer to expose the structures underneath and then visualized them with scanning electron microscopy (SEM). In our study, we used histology rather then SEM. Second, unlike Shardo (1995), we could not define the same steps of tail and head lifting, because the developmental rate of the cichlids was faster.

Similar issues arise if we compare our stages to those of Kimmel et al., (1995) for the zebrafish, *Danio rerio*, a cyprinodont. That study divided de-

velopment into a series of periods, each of which was further subdivided on the basis of the state of one or two characters (e.g., somites or pectoral fin).

Stages may also be defined according to chronological age. Balon (1985) divided development of the cichlids *Labeotropheus trewavasae* and *L. fuelleborni* into phases that were further subdivided according to chronological age of the embryos. Fishelson (1966) described stages of development in two mouth-brooding and one substrate-breeding cichlid, based on chronological age, for various organ systems, without giving "whole body" stages.

As can be seen in Figure 12, different authors may divide the same developmental sequence into different numbers of stages (represented in the charts by vertical lines). The wider the stages are spaced, the lower the temporal resolution of that series (i.e., the ability to resolve two events as non-simultaneous).

Character Homology

Many character states used for staging are in fact developmental transformations (*sensu* Bininda-Emonds et al., 2002); that is, transformations between two character states. This is inevitable, given that development is a continuous process. Unfortunately, however, there is no standardized methodology for homologizing developmental transformations. For example, a commonly used character state in stage definitions is *pectoral fin bud*. In principle, however, it may not be obvious exactly when the pectoral fin should be called a bud. It could be when it is visible as a mesenchymal cell condensation, or when it starts to protrude from the surface of the yolk sac. There is also a multitude of synonyms used in developmental biology. For the developing eye, for example, possible terms includes the following: optic Anlage, optic bud, optic outgrowth, optic vesicle, optic diverticulum, eye primordium, and so on. This structure is even called *optic lobe* (Swarup, 1958, p 379) even though most authors, including ourselves reserve that term for the optic lobes of the midbrain. Such issues are part of wider problem of an inconsistent use of standardized nomenclature in developmental biology.

Other problematic characters are those that can have multiple definitions. An example is the character *brain primordium* or *brain rudiment* (used by Kimmel et al., 1995, p 282; Morrison et al., 2001, p 178). Possible definitions include i) brain distinct from spinal cord (Swarup, 1958, p 379); ii) neural keel slightly broader at cephalic end (Tavolga, 1949, p 173); iii) primordium of the brain is visible (Iwamatsu, 2004, p 607; Fujimura and Okada, 2007, p 308). Another character that is often weakly defined is the unpaired median fin fold. The appearance of this character is variously defined in terms of fin fold (Shardo, 1995, p 143), tail fin (Swarup, 1958, p 380) or membranous fins (fin fold) (Iwamatsu, 2004, p 612). Many clearly defined characters by contrast (e.g., *tailbud*, *rhombomeres*, *olfactory placodes*) are unfortunately not described in all studies. In the future, it is hoped that staging systems can be based on a standardized set of robustly defined characters.

Heterochrony

Yet another factor that can lead to lack of concordance between staging series is *heterochrony*, i.e., a genuine biological difference in the sequence or timing of developmental events. Heterochrony and its biological implications have been considered extensively elsewhere (Gould, 1977; Richardson, 1995; Klingenberg, 1998; Richardson, 1999; Mabee et al., 2000; Smith, 2001; Jeffery et al., 2002; Richardson and Oelschäger, 2002). Jones (1974) noted that heterochrony causes problems in comparison between different substrate brooding cichlid species and species hybrids. He did not find significant differences between the developmental characters themselves in these groups, but there were remarkable differences in the relative timing (i.e., sequence) of developmental characters.

Heterochrony has been noted for larger taxonomic groups in the vertebrates (e.g., Richardson, 1995) and is illustrated here for our species and other selected teleosts in Figure 12. As can be seen, the character sequence [embryonic shield → optic Anlagen → auditory vesicles] is always constant. Other characters, however, show examples of heterochrony. The character pair heartbeat and auditory vesicle are simultaneous in our study, in the blackchin tilapia, and in the substrate brooding cichlids (Fig. 12A,C,D). By contrast, heartbeat develops later than auditory vesicle in *Oreochromis niloticus*, stickleback, platyfish, Atlantic killifish, medaka, zebrafish, and American shad (Fig. 12B,E–J). The character pair pectoral fin bud and retinal pigmentation are simultaneous in our study and in the Atlantic killifish (Fig. 12A,G); however, the pectoral fin bud appears earlier than retinal pigmentation in *O. niloticus*, platyfish, and American shad (Fig. 12B,F,J). The pectoral fin bud develops later than retinal pigmentation in the blackchin tilapia, substrate brooding cichlids, stickleback, medaka, and zebrafish (Fig. 12C–E,H,I). The heartbeat develops before the pectoral fin bud in our study, *O. niloticus*, blackchin tilapia, substrate brooding cichlids, stickleback, Atlantic killifish, medaka, and zebrafish (Fig. 12A–E,G–I), whereas in the platyfish and in the American shad, this timing relationship is reversed (Fig. 12F,J).

In summary, we have described a normal series of stages for *Haplochromis piceatus* based on key characters. We have compared our data with published staging series for other teleosts, and found

significant differences between them. We found that none of the published staging series can be easily applied either to our cichlid, *Haplochromis piceatus*, or to any from a wide range of fish species. This lack of concordance shows the importance of determining staging series for each taxonomic group studied. The East African haplochromine cichlids are of great environmental, biological, and societal importance. Therefore, we hope that our study, the first staging series for a haplochromine cichlid, will be useful for research in this field.

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