

**Early development of the self-fertilizing mangrove killifish, *Rivulus marmoratus*,  
reared in laboratory**

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**Abstract** The mangrove killifish, *Rivulus marmoratus*, was reared at  $25 \pm 1^\circ\text{C}$  and 17 ppt salinity from 0 to 100 days after hatching (DAH), and its early development was described by examining growth and morphometric parameters, meristic characters (vertebral and fin-ray counts), bone-cartilage development, and pigmentation. Growth was isometric for preanal length, head length, snout length, body depth, pectoral-fin length, dorsal-fin length, anal-fin length, and caudal-peduncle depth. Negative allometric growth was observed in eye diameter and gape size. Meristic counts (mean  $\pm$  SD) for vertebrae ( $34.2 \pm 0.4$ ), dorsal- ( $8.6 \pm 0.5$ ), anal- ( $11.4 \pm 0.5$ ), and caudal-fin rays ( $30.2 \pm 0.8$ ) were complete at 0 DAH ( $n = 5$ ), while pectoral-fin rays and pelvic-fin rays were complete by 30 DAH ( $14.5 \pm 0.4$ ,  $n = 5$ ) and 60 DAH ( $4.2 \pm 0.8$ ,  $n = 5$ ). Full ossification of meristic elements proceeded in the following sequence: vertebrae (by 30 DAH), caudal-, dorsal-, and anal-fin rays (by 60 DAH), pectoral-fin rays (between 60 DAH and 100 DAH), and pelvic-fin rays (by 100 DAH). Both morphological characters and meristic counts indicate that this species can be considered to be a juvenile after 9.8 mm in standard length (20 DAH).

**Key Words** *Rivulus marmoratus* · Morphometry · Ossification · Ontogeny · Mangrove killifish

Mangrove killifish, *Rivulus marmoratus* (family Rivulidae) inhabit mangrove areas, and are widely distributed from Brazil to Florida (Davis et al., 1990; Taylor et al., 1995). In the wild, the adults grow up to a maximum size of over 45 mm in total length (Davis et al., 1990). This species is well known to be a model species among marine fishes because of its unusual reproductivity (internal self-fertilization), ease of culture, and high sensitivity to toxic materials (Harrington, 1961; Koenig and Chasar, 1984; Abel et al., 1987). According to Harrington (1975) and Soto et al. (1992), three sexual phenotypes are recognized in laboratory-reared individuals: hermaphrodites (body with marbled brownish color pattern usually with caudal ocellus); primary males (develop directly from juveniles, with orange body coloration usually without caudal ocellus that produce only sperm); and secondary males (arise from hermaphrodites following the loss of female function). The hermaphrodites are capable of internal self-fertilization, producing clonal lineages (Kallman and Harrington, 1964; Harrington and Kallman, 1968).

Although *Rivulus marmoratus* has often been used as an experimental animal for population and genetic (Turner et al., 1992), toxicity (Park et al., 1994), and carcinogenicity studies (Koenig and Chasar, 1984), there are no comprehensive studies on the ontogenetic development of larvae and juveniles. Morphological studies are

important, because modification or transformation of an organism is usually in preparation for a change in environment, behavior, or mode of feeding. Conversely, behavioral modifications are associated with morphological and physiological changes (Yuoson, 1988).

Most of the previous studies on the morphology of *R. marmoratus* were focused on characters at certain developmental stages such as embryonic stage: developmental instability of meristic features as induced by temperature (Lindsey and Harrington, 1972; Harrington and Crossman, 1976); embryonic development for establishing its use for carcinogenicity tests (Koenig and Chasar, 1984); and development of retinal pigmented epithelium (Ali et al., 1988a, 1988b, 1989). In the adult stage, skeletal system (Lee and Park, 1989), pseudobranch structure (King et al., 1993), morphology and function of internal organs (Thiyagarajah and Grizzle, 1995) have been described. Spontaneous bilateral asymmetry (Park et al., 1987), scale morphology and growth (Park and Lee, 1988), and aging by otolith increments and early sexual development (Sakakura and Noakes, 2000) from larvae to early adult stage have also been studied. Our study describes the sequential development of *R. marmoratus* with emphasis on morphometric and meristic features, ossification, and pigmentation at different stages (0-100 days after hatching: DAH). A clear definition of characters of each

developmental stage is relevant because it may be correlated with their behaviors such as feeding, swimming, and other aspects of life history.

## **Materials and Methods**

Experimental materials. — PAN-RS strain derived from reared broodstock was used, which originated from Florida, U.S.A. and obtained through Dr. W.P. Davis (U.S. Environmental Protection Agency, Gulf Breeze, Florida). This strain was originally collected in Florida in 1994, and has been successfully reared for over 5 generations in our laboratory.

A total of 65 eggs at stage 32 (final stage before hatching, characterized by opening and closing of mouth with opercular movement, motile pectoral fins, ray formation in dorsal and anal fins, and appearance of pigmentation along the rays of caudal and pectoral fins; see Koenig and Chasar, 1984) were manually dechorionated by the removal of chorion/egg case to allow for the release of larvae using fine forceps, following the protocol of Koenig and Chasar (1984). The larvae were individually reared in plastic cups (5.8 cm diameter) filled with 60 ml of 17 ppt artificial brackish

waters (Marine Art Hi, Tomita Pharmaceutical Co. Ltd., Naruto), previously used by Sakakura and Noakes (2000), at  $25 \pm 1^\circ\text{C}$  and 14L:10D photoperiod. Rearing cups were washed and water was replaced every two weeks. The larvae were fed with newly hatched *Artemia franciscana*, to maintain 8 nauplii/ml every two days.

Morphometric measurements. — A total of 65 specimens were sampled; 5 specimens on 0 and 5 DAH; 10 specimens on 10, 20, 30, and 60 DAH; and 15 specimens on 100 DAH, respectively. The specimens were anaesthetized with over 400 ppm MS 222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis) and were immediately fixed in 5 % formalin solution. Scientific drawings were made using a dissecting microscope (Olympus SZX12) with camera lucida at 20 to 40x magnification. We examined the following morphological characters: total length (TL), standard length (SL), preanal length (PAL), head length (HL), eye diameter (ED), snout length (SnL), gape size ( $\text{GS} = \sqrt{2} \times \text{upper jaw length (UJL)}$ , Shirota, 1970), body depth (BD), dorsal-fin length (DL), pectoral-fin length (PcL), pelvic-fin length (PvL), anal-fin length (AL), and caudal-peduncle depth (CPD) [measured to the nearest 0.01 mm using a digital microscope (VH 6300, Keyence Corp., Japan)]. These measurements follow McAllister and Smith (1978) and Strauss and Bond (1990), except DL and AL, which were measured from the origin of the fin base to the

posterior-most portion of the fin margin.

Ossification.— Bone-cartilage development and meristic characters were examined in 5 specimens on 0, 5, 10, 20, 30, 60, and 100 DAH. A clearing-staining technique was used following the modified procedure of Potthoff (1984). The number of ossified and non-ossified vertebrae and fin rays was counted under a dissecting microscope. Ossification of skeletal parts was described following the terms used by Lee and Park (1989).

Statistical analyses. — Growth expressed as SL plotted over time (0 - 100 DAH) was fitted to the Gompertz growth equation:  $Y = ae^{-e^{-\frac{(X-X_0)}{b}}}$ , where  $Y = \text{SL}$ ,  $X = \text{DAH}$ ,  $X_0 = \text{initial instantaneous growth rate}$ ,  $a = \text{slope}$ , and  $b = \text{Y-intercept or the theoretical length which corresponds to age 0}$  (Gamito, 1998) using Sigma Plot version 8.0 (SPSS Inc.). Morphometric measurements were analyzed following the method used by Masuda and Tsukamoto (1996). This was done by fitting the measurements to the power equation:  $Y = aX^b$ , where  $Y = \text{morphometric measurements}$ ,  $X = \text{standard length}$ , and  $b = \text{slope}$  using Sigma Plot version 8.0 (SPSS Inc.). When  $b$  was equal to 1, growth was considered isometric,  $b < 1$ , negatively allometric, and  $b > 1$ , positively allometric. To test whether  $b$  deviated significantly from 1,  $t$ -test of Clarke (1980) was used (Aguirre, 2003; Borja, 2003). When  $b$  significantly deviated from 1, it was

considered as either positively allometric ( $b > 1$ ) or negatively allometric ( $b < 1$ ).

When no significant difference was found, the relationship was considered isometric.

Actual measurements were used instead of ratios, because the latter gives inaccurate results and may often lead to erroneous interpretations (Marr, 1955).

## Results

**Early growth.** — The body size in terms of SL during early growth ranged from  $4.4 \pm 0.1$  mm (mean  $\pm$  SD,  $n = 5$ ) at 0 DAH to  $16.6 \pm 1.1$  mm ( $n = 5$ ) at 100 DAH. SL and DAH relationship was closely fitted by the Gompertz growth equation (Fig. 1,  $r^2 = 0.98$ ). Growth rate was rapid until 20 DAH, and then started to level off until 100 DAH.

Results of the power function relationship for morphometric values showed isometric growth for PAL ( $b = 1.032$ ), HL ( $b = 0.9438$ ), SnL ( $b = 1.082$ ), BD ( $b = 0.9289$ ), PcL ( $b = 1.052$ ), DL ( $b = 1.049$ ), AL ( $b = 1.034$ ), and CPD ( $b = 1.120$ ) ( $t$ -test of  $b = 1$ ,  $t < 4.303$ ,  $P > 0.05$ ). Negative allometric growth was observed for ED and GS ( $b$  values were 0.8325 and 0.4559,  $t$ -test,  $t = 4.303$ ,  $P \leq 0.05$ ), while strong positive

allometric growth for PvL ( $b = 2.962$ ,  $t$ -test,  $t = 6.965$ ,  $P = 0.02$ ). However, PvL showed a weak relationship with SL ( $r^2 = 0.6$ ), therefore no comments could be made regarding its growth (Table 1).

**Pigmentation.** — Of the 65 specimens examined, 45 did not show the orange coloration on body and fins. Out of these 45 specimens, 33 had caudal ocellus ( $\geq 20$  DAH, Fig. 2D,E) while the remaining had none. The caudal ocellus is present in typical hermaphrodites, and a weak correlation is known to exist between gonad and external appearance (see Soto and Noakes, 1994, but they could not make any comments on the sexuality).

Pigmentation generally appeared as brownish mottled brown in all specimens, however, concentrated in different areas throughout development (Fig. 2). The head of newly hatched larvae had few, yet concentrated melanophores dorsally, and around mouth and on snout (Fig. 2A). Melanophores were present postero-ventrally around eye and on opercule, the latter became sparse at juvenile stage (Fig. 2D,E). Newly hatched larvae were heavily pigmented along dorsal midline and midlaterally on body (Fig. 2A), both of which became sparsely scattered on body by 20 DAH (Fig. 2D). Finally, it became evenly and thinly distributed, appearing mottled by 30 DAH. The ocellus began to form by 20 DAH and was always present in all specimens until 100

DAH except in 12 specimens at 100 DAH. Melanophores located ventrally on the gut were absent by 20 DAH (Fig. 2D). Scale formation started at the center of the parietal region at 10 DAH.

**Meristic counts and ossification of vertebrae and fin rays.** — Vertebral count (mean  $\pm$  SD,  $34.2 \pm 0.4$ ,  $n = 5$ ) was complete in newly hatched larvae. Ossification started at 5 DAH ( $1.5 \pm 0.8$  in 4 of 5 specimens), and then full ossification was attained by 30 DAH ( $33.0$  in 5 of 5 specimens, Fig. 3A). Counts for dorsal- ( $8.6 \pm 0.5$ ,  $n = 5$ ), anal- ( $11.4 \pm 0.5$ ,  $n = 5$ ), and caudal-fin rays ( $30.2 \pm 0.8$ ,  $n = 15$ ) were complete in newly hatched larvae (Fig. 3C,E,F). Their ossification varied; both dorsal- and anal-fin rays started to ossify from 30 DAH ( $2 \pm 2.8$  in 2 of 5 specimens, and  $2.4 \pm 3.6$  in 2 of 5 specimens, respectively) with complete ossification attained by 60 DAH (respective counts were  $7.6 \pm 0.6$  in 5 of 5 specimens and  $11.6 \pm 0.89$  in 4 of 5 specimens, Fig. 3C,E). Caudal fins started to ossify at 5 DAH ( $11.4 \pm 0.6$  in 5 of 5 specimens) and full ossification was achieved by 60 DAH ( $30.6 \pm 0.6$  in 5 of 5 specimens, Fig. 3F). Pectoral-fin rays (left and right) had complete count by 30 DAH ( $14.5 \pm 0.4$ ,  $n = 5$ ), but ossification started at 30 DAH ( $2.5 \pm 4.1$  in 2 of 5 specimens) and then was completed by 60 DAH ( $12.3 \pm 0.5$  in 5 of 5 specimens, Fig. 3B). Pelvic-fin rays appeared at 20 DAH ( $2.6 \pm 1.5$ ,  $n = 4$ ) with complete fin-ray count

observed by 60 DAH ( $4.2 \pm 0.8$ ,  $n = 5$ ). Both initial and full ossification was attained by 100 DAH (5.0 in 5 of 5 specimens, Fig. 3D).

**Skull ossification.** — Ossification of the subopercule started at 5 DAH (Fig. 4A). At 20 DAH, the largest variation on the ossification was observable: frontal, parietal, sphenotic, pterotic, epiotic, supraoccipital, exoccipital, and parasphenoid not ossified in 2 specimens but ossified in 3 specimens; gill arches, opercule, premaxilla, maxilla, dentary, and anguloarticular ossified in 2 but not ossified in 3 (Fig. 4B). By 30 DAH, all parts of the skull were ossified except cartilaginous nasal (not all but small part), opercule, dentary, and anguloarticular (Fig. 4C). At 60 DAH onwards, complete ossification was attained (Fig. 4D).

## **Discussion**

**Early growth, external appearance, and gender.** — Growth curves were similar with previous reports (Park et al., 1987; Park and Lee, 1988; Koenig and Chasar, 1984; Sakakura and Noakes, 2000), but some differences were found in growth rate. Park and Lee (1988) reported size range as 7 - 20 mm TL (7 - 84 DAH). In this study, although no measurements were taken at 84 DAH, it could be inferred from the

measurements derived at 100 DAH that 20 mm TL was achieved at a later age. The temperature used was similar in both Park and Lee's (1988) study and ours, but the differences may be due to dissimilarities in strain and rearing conditions [e.g., salinity and culture containers (parental stock obtained from Germany, 15 ppt, and 1-l glass chamber/fish, respectively)]. TL of newly hatched larvae in the present study (mean TL  $\pm$  SD =  $5.6 \pm 0.1$  mm) was close to that of the length reported by Sakakura and Noakes (2000) who used the same strain and rearing conditions.

Physical appearance (e.g., non-orange fish with dark caudal ocellus for hermaphrodites and orange coloration with no distinct caudal ocellus for males) has been used previously to describe gender (Harrington, 1975). Based on histological analysis of gonads, more than half of specimens observed do not fall into male-hermaphrodite categories (Soto and Noakes, 1994). They emphasized that absence or faded appearance of caudal ocellus alone was not a reliable indicator of gender, neither was the degree of orange coloration. Therefore, neither external character should be considered independently in determining sexuality. The absence of correlation between sexual development and coloration during early life of this species had also been suggested (Sakakura and Noakes, 2000). Based on previous arguments correlating body color and gender, it is, therefore, difficult to comment on the sexuality

of the specimens studied, considering that they may still be juveniles.

**Attainment of juvenile stage.** — The juvenile stage is characterized by having the appearance of small adults; all fin rays and scales are formed, the skeleton is almost completely ossified, the larval pigment is overgrown and replaced by dermal pigments similar to that of adults, and the body shape approximates that of adults (Kendall et al., 1984). Based on this definition, the major inflection in growth may indicate that the juvenile stage occurs after 20 DAH (12.0 mm TL, 9.8 mm SL). Since sampling was not done between 10 and 20 DAH, it may be possible that this has even started earlier, as what has been observed in a previous study (Park and Lee, 1988), which has shown that scale formation (squamation) starts at 8 DAH (7.1 mm TL). In this study, scale formation started at 10 DAH. Disparity in results may be due to differences in the parental stock (obtained from the Zoology Institute and Museum, University of Hamburg, Federal Republic of Germany in 1981) and rearing conditions, such as salinity (15 ppt) and size of rearing tanks (1-l glass chambers) used by Park and Lee (1988), whereas, 17 ppt and 60 ml plastic cups were used in this study. On the other hand, complete fin-ray counts and ossification were attained by 100 DAH. All medial fins and pectoral fins were completed by 60 DAH while the pelvic fins at 100 DAH. Ossification of vertebrae was completed by 30 DAH, while the skull by 60 DAH. Full

ossification of all fins was attained by 100 DAH. Based from these observations, it can be deduced that the juvenile stage occurs after 20 DAH until 100 DAH.

**Meristic counts.** — Vertebral count was complete in newly hatched larvae, which coincided with Lindsey and Harrington (1972). Dorsal- and anal-fin ray counts also corresponded with ranges observed in embryos, 8-11 and 11-13, respectively (Lindsey and Harrington, 1972). Vertebral counts, dorsal-, anal-, caudal- and pectoral fin-ray counts (respective means were 33.6, 8.8, 11.5, 30, 13.9) reported by Swain and Lindsey (1986) corresponded with our results. Pectoral- and pelvic-fin ray counts ( $13.0 \pm 0.5$  and  $5.8 \pm 1.6$ ) in 90 to 100 days post-hatched individuals reported by Park et al. (1987) were close to the counts in our observation ( $14.5 \pm 0.5$  and  $5.0 \pm 0.0$ , respectively). But a difference in pectoral-fin ray count (= 1) may be due to different salinity between the previous studies and ours ( $10 \pm 1$  ppt), although this speculation needs further confirmation. Previous studies reported the meristic characters at certain developmental stages only (see Lindsey and Harrington, 1972; Swain and Lindsey, 1986; Park et al., 1987). Our study revealed sequential meristic counts at various stages of development, showing that complete counts of vertebrae, dorsal-, anal-, and caudal-fin rays were attained even before hatching, and no significant change in counts was observed between 0 DAH (4.4 mm SL) and 100 DAH (16.6 mm SL). Fins were

still developing and becoming ossified. Pectoral-fin ray count was completed from 60 until 100 DAH, while for pelvic-fin rays by 100 DAH. The completion of pectoral and pelvic fin ray counts at later stages of development may indicate their use at later stages and that they are still juveniles. Pectoral fins have been reported to have a major role in maneuverability (Lindsey, 1978). Pelvic fins on the other hand, formed late in development, thus, grew at a relatively fast growth once it was formed. This explains the poor fit in the regression observed and its strong allometry.

With some exceptions, pectoral fins have been known to develop rays later in the larval period than median fins (Dunn, 1984), which is consistent with the ossification sequence observed in this study.

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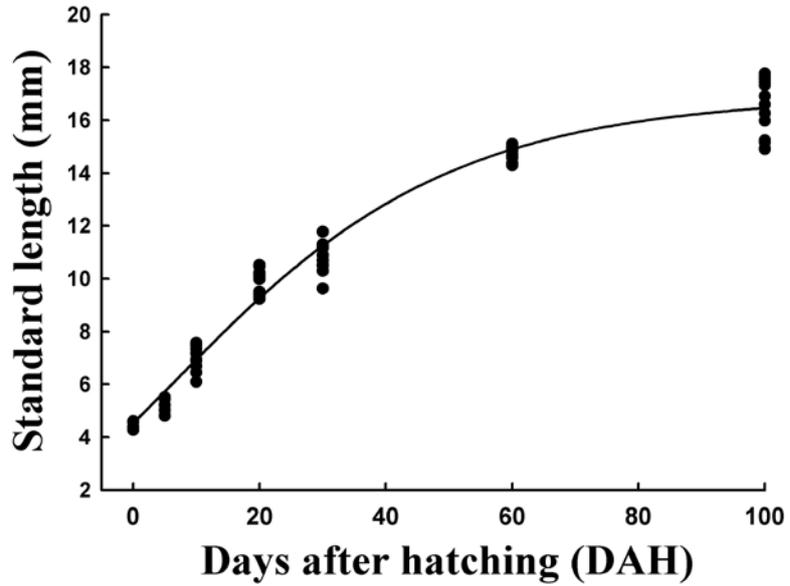
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**Table 1.** Coefficients of regression analysis of the morphometric variables measured in *Rivulus marmoratus* with corresponding  $a$ ,  $b$ , and  $r$  values ( $Y = aX^b$ ;  $n = 65$ ;  $P < 0.001$  for all variables)

<b>Morphometric variables</b>	<b>a</b>	<b>b</b>	<b>r</b>
Preanal length	0.5659	1.032	0.99
Head length	0.3161	0.9438	0.97
Eye diameter	0.1231	0.8325 <sup>a</sup>	0.93
Snout length	0.0582	1.082	0.88
Gape size	0.3665	0.4559 <sup>a</sup>	0.84
Body depth	0.2423	0.9289	0.96
Pectoral-fin length	0.1480	1.052	0.94
Pelvic-fin length	0.0002	2.962 <sup>a</sup>	0.78
Dorsal-fin length	0.1461	1.049	0.94
Anal-fin length	0.1914	1.034	0.96
Caudal-peduncle depth	0.0913	1.122	0.96

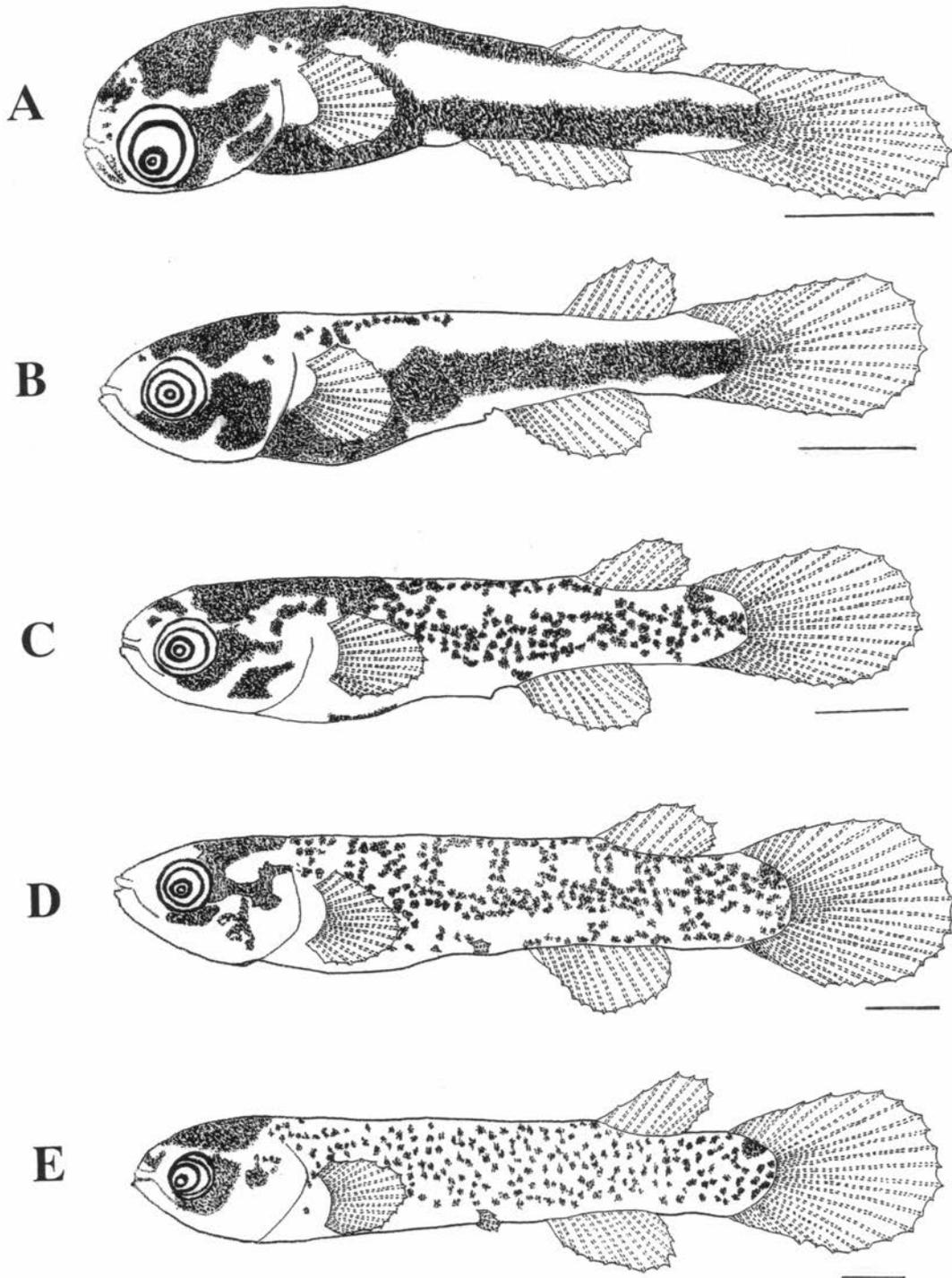
<sup>a</sup> Significantly different from  $b = 1$ ,  $t \geq 4.303$  at  $P \leq 0.05$  ( $t$ -test)

## Figure Legends

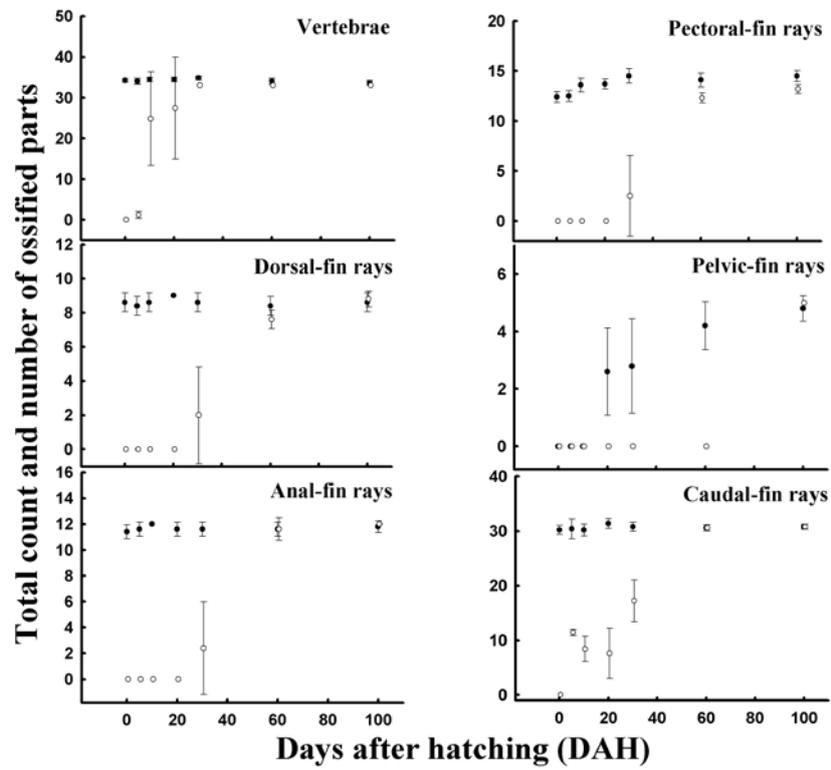


**Fig. 1.** Growth curve expressed as standard length (SL) during early development of

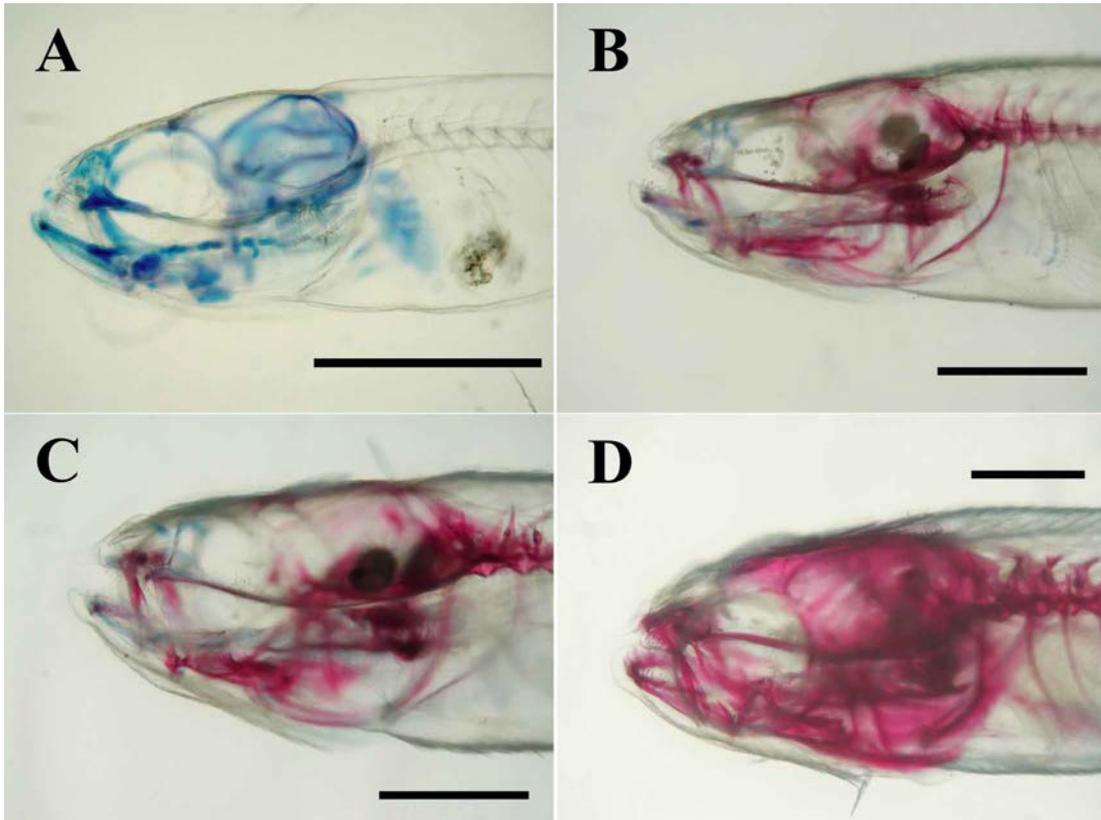
$$Rivulus marmoratus (Y = 16.9e^{-e^{-\frac{(X-6.70)}{25.6}}}; n = 65; r^2 = 0.98)$$



**Fig. 2.** Morphological development of *Rivulus marmoratus*. **A** Newly hatched larvae, 4.4 mm SL. **B** 5 DAH larva, 5.7 mm SL. **C** 10 DAH larva, 6.7 mm SL. **D** 20 DAH juvenile, 9.8 mm SL. **E** 30 DAH juvenile, 10.9 mm SL. *Bars* indicate 1 mm



**Fig. 3.** Vertebral and fin-ray counts (*closed circles*) and ossification (*open circles*) (mean  $\pm$  SD) from 0 to 100 DAH in *Rivulus marmoratus*



**Fig. 4.** Ossification of skull of *Rivulus marmoratus*. **A** 5 DAH, 4.2 mm SL. **B** 20 DAH, 10.0 mm SL. **C** 30 DAH, 11.8 mm SL. **D** 60 DAH, 15.1 mm SL. *Bars* indicate 1 mm