Embryogenesis and early larval development in rosy barb (*Pethia conchonius*, Hamilton 1822)

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ABSTRACT

This study aimed to investigate the larval ontogeny of rosy barb *Pethia conchonius* (Hamilton 1822). Embryonic and larval development stages were studied using the microscopy technique. Trials were carried out on 60 broodstock of rosy barb. Round and transparent fertilized eggs with a diameter of 867.6 ± 21.7 µm have started to hatch approximately 27 h after spawning at 26.3°C. The newly hatched larvae (n:30) have a mean total length of 4227 ± 265 µm and started exogenous feeding 4 days after hatching (DAH) and started taking particulate and powder feed at the end of 18 DAH. The early development growth formula of rosy barb has an exponential relationship model of $y = 3.8346e^{0.0313x}$ ($R^2 = 0.9112$, n = 140). Digestive tract differentiation, hepatopancreas, digestive tubes, and gill arches formations were examined by taking histological sections in the early life stages of the rosy barb. Rosy barb is a species whose production protocol is not difficult due to its short egg hatching period and early larval stage zooplankton feeding period.

Keywords: Cyprinidae, fish larvae, microscopy, ontogeny, ornamental fishes

INTRODUCTION

Ornamental aquaculture, once a hobby, has now evolved into a significant agricultural sector with a global appeal, attracting millions of enthusiasts and generating substantial economic returns. This growth has led to the emergence of major large-scale producers of aquarium fish in many countries (Hekimoğlu 2006). The increasing species diversity in the aquarium sector, coupled with the demand for equipment and maintenance materials, has created a new commercial field, further expanding the sector's trade volume on a global scale (Reid et al. 2013; Hunt and Koca 2014).

In aquarium fisheries, many species belonging to different families are used in aquariums prepared with various concepts. Increasing the diversity of aquarium fish species and introducing new

species by determining breeding procedures is very important for the sector. Expanding the breeding of ornamental fish species instead of hunting them from the natural environment is crucial in reducing hunting pressure in natural populations. Ontogeny studies are essential in determining rearing and larval feeding procedures. Therefore, this study aimed to reveal the embryogenic and larval development of an ornamental fish belonging to the Cyprinidae family. Cyprinidae is one of the most important families, and it includes many economical and popular species used as ornamental fish, represented by 161 genera and 1727 species worldwide (Fricke et al. 2024). Although they are generally freshwater species, they are also distributed in brackish waters and marine coastal areas (FishBase 2022). A member of the Cyprinidae family, the rosy barb, was used in this study. The rosy barbs are popular among aquarium fishes due to their visual beauty and compatibility with other small fishes.

Rosy barbs are typical in Asia, specifically in Afghanistan, Pakistan, India, Nepal, Bangladesh (Talwar and Jhingran 1991), and Myanmar (Oo 2002). Individuals of this species can reach a maximum length of 14 cm, and the female is larger than the male (Talwar and Jhingran 1991). Male of this species, typically silver, take on a striking claret-red color, especially during the breeding season (Mills and Vevers 1989; Allen et al. 2002). Their food comprises worms, crustaceans, insects, and plant matter (Allen et al. 2002).

The highest losses in the culture of ornamental fishes occur in the early larval stages. For this reason, knowing the larval development stages is imperative. Fish larvae are generally transparent in their developmental processes up to the postlarval stage, allowing microscopes to be used in ontogeny studies (Sepil et al. 2022). Examination of various tissues and organs formed due to the development of the larvae generally necessitates the use of histological methods. It is essential to use microscopy methods to determine the early larval feeding procedures, monitor metamorphosis, and determine the stages during mouth opening, swim bladder formation, differentiation of the digestive tract, and formation of a functional stomach.

In this study, embryonic and early larval development and allometric growth measurements of rosy barbs were examined microscopically. In this regard, it is aimed to reveal important metamorphosis stages such as the structure of eggs, hatching time, yolk sac absorption time of larvae, exogenous feeding time, determination of larval feeding protocol, development of tissues and organs and their transformation into functional structure.

METHODS

The broodstocks used in this study were obtained from adult rosy barbs (from Van Yüzüncü Yıl University, Faculty of Fisheries, Aquatic Creatures Experimental Unit, Türkiye) with a total length of >4 cm. A set of broodstocks (n: 6) were placed into separate aquariums with 50 L volumes according to identified sexes, where each aquarium was stocked with 5 female and 5 male broodstocks. Commercial flake and granule feeds were used at certain rates (10% Tetra discus granule and 90% Tetra pro-energy flake) in feeding approximately 4% of body weight twice a day (Gosh et al. 2008). Newly hatched Artemia sp. nauplii and bloodworms were fed to adapt the fishes to granular and flake feeds. The average water temperature in aquariums was 26.11 ± 1.16°C, pH at 8.12 ± 0.27, electrical conductivity (EC) at 851 ± 114.6 µS/cm, salinity at 0.41 ± 0.13 mg/L, and dissolved oxygen (DO) at 7.22 ± 0.15 mg/L during the acclimation process. Eggs and larvae of rosy barbs, produced after a certain conditioning period, were examined under the microscope. Then, histological examination was performed after the samples were prepared using appropriate fixative and alcohol series, and sections were taken from paraffin blocks.

Spawning of Broodstock Rosy Barbs

Breeding was carried out in 40 L glass aquariums with a water temperature of 26.3 ± 0.4°C, EC at 49.1 ± 3.2 µS/cm, and pH at 6.90 ± 0.10 after 2 weeks of conditioning. The broodstocks were placed into 1 cm mesh cages with 1 female and 1 male fish to prevent egg predation. After the eggs were observed on the aquarium floor, the broodstocks were removed from the tank, and egg sampling was carried out.

Egg and Larva Sampling and Preparations

The day after hatching (DAH) occurred was considered the first day of the larvae. Egg samples taken from the same broodstock rosy barb in a single batch were sampled at 6, 12, and 24 hours and just before egg hatching. Furthermore, larvae (n: 30) were sampled every day in the first 10 days and once every 2 days from the 10th day until the 50th day. Ten randomly selected eggs and larvae were fixed with Bouin's solution (Sigma-Aldrich, USA) during each sampling time. Eggs and larvae were first soaked in 250 mg L^{-1} of Tricaine Methanesulfonate (MS-222) for euthanasia for about a few minutes (AVMA 2007; Topic-Popovic et al. 2012) and then fixed in Bouin's solution (Moore et al. 2002; Ünal 2010) for 24 hours at room temperature. Following the dehydration process in the alcohol series, the samples taken into base molds were embedded in paraffin. Sections with

a thickness of 5 µm were obtained from paraffin blocks using the rotary microtome device (MICROM HM 315, Walldorf-Germany) (Onal et al. 2008; Santos et al. 2016). Thin sections (5 µ) with Haematoxylin-Eosin and Trichrome staining were examined under a light microscope to determine embryonic and larval developmental stages (Onal et al. 2008 Santos et al. 2016; Aminaghaie and Esmaeili 2017). Morphometric measurements of samples were performed using ImageJ 1.46r software (Onalan and Sepil 2024). The first egg diameter, the number of oil droplets, the pigmentation pattern seen in the egg and larva, the egg hatching period and developmental stages, the first larva size, the absorption time of the yolk sac, the opening of the mouth and anus and the length of the first mouth opening were studied. Mouth gap sizes were examined in fish larvae when the first exogenous feeding begins; the vertical distance between the jaws and the distance between the horizontal joints were measured at the point where the mouth was fully opened (Ramezani Fard et al. 2011; Riar et al. 2018). Allometric growth parameters for total length (TL), head length (HL), tail length (TLL), eye diameter (ED), Pre-anal myomere length (PrAM) and Post-anal myomere length (PoAM) changes were determined for the samples (Figure 1).

Allometric growth models are defined by linear regression formulas, which are determined by associating related body regions with total length (TL) (Gisbert et al. 2002; Çelik et al. 2011; Sepil et al. 2022). According to total height ratios, meristic growth characters such as HL, TLL, ED, PrAM, and PoAM were estimated using the allometric equation below;

$$Y = a \times (W^b)$$

Y = Measured character, W = Independent variable (TL), a = Intersection point, and b = Growth coefficient (Gisbert et al. 2002).

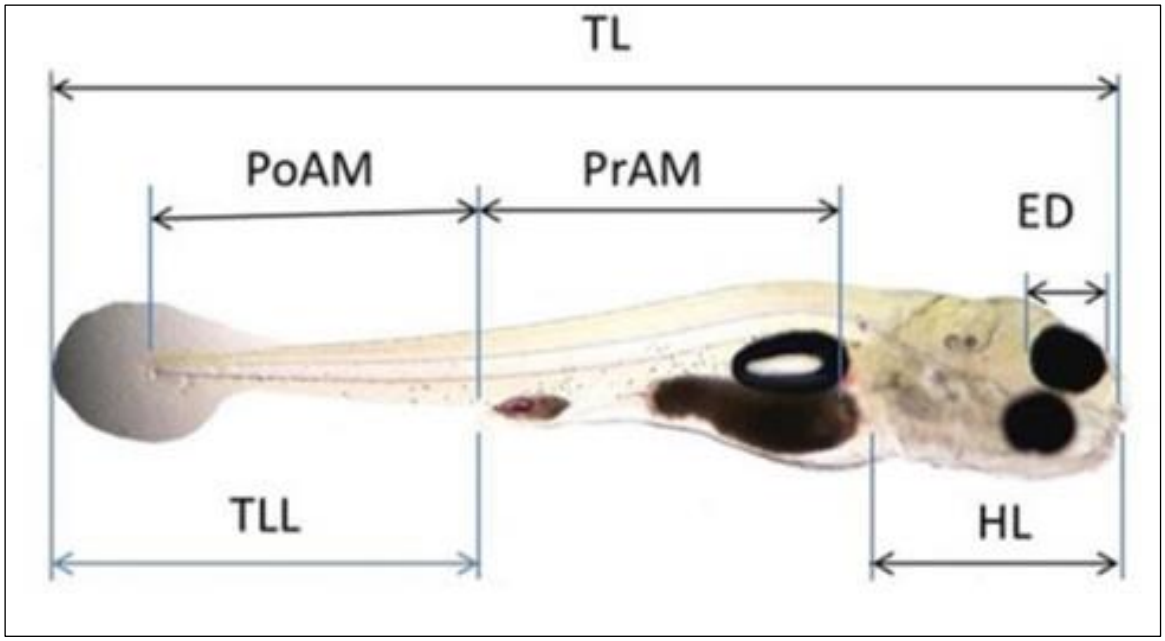

Figure 1. Some meristic growth characters on larvae (TL: total length, PoAM: Post-anal myomere length, PrAM: Pre-anal myomere length, ED: eye diameter, TLL: tail length, HL: head length).

RESULTS

Spawning and Egg Characteristics

It has been observed that the movements of female slow down considerably during reproduction and before laying eggs, while male is more active throughout the entire water column. However, it was observed that the colors of the female were brighter and darker red before breeding, while there was no change in the coloration of the male.

The experimental broodstocks were observed to spawn 12 times, and spawning was carried out at different times from each group during the trial. Different female individuals were used in each breeding process. The female broodstocks with the fullest abdomen from the stock aquariums were taken

into the breeding cage. Eggs were observed on the ground 12-15 hours after the broodstock were placed in the production cages. For the eggs to be fully fertilized, the distance between the production cage floor and the aquarium bottom was kept short, and after spawning was completed, the broodstock was kept in the cage for another 2 hours. In all breeding trials, fecundity was determined to be 224 ± 13 eggs/female. The eggs were observed to be round in shape and transparent (Figure 2). The samples examined within the first 3-6 hours from the release of eggs showed that the egg diameter was in the range of 646-1457 µm, and the average diameter was 867.6 ± 21.7 µm (n: 120). It was found that the water temperature values directly affect the egg hatching time in the decapsulation of eggs. In trials carried out at different temperatures, the relationship between temperature and egg hatching time is given in the graph in Figure 3.

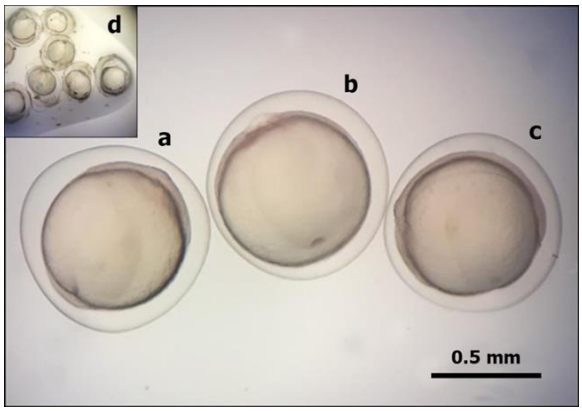

Figure 2. Shape and structure of eggs in different stages (a: 2 somite formation, b: Sphere stage, blastodisc flattened and multi-cell formation, c: embryo formation, d: embryo formation, pre-hatching).

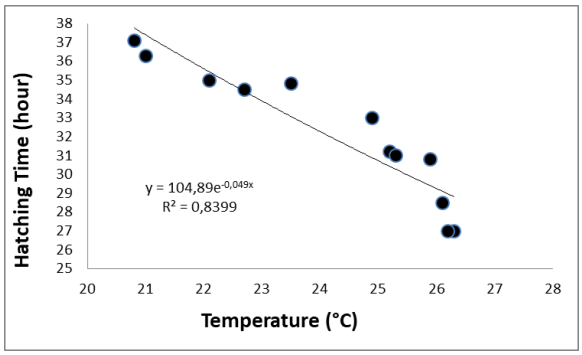

Figure 3. Relationship between temperature and egg hatching time in rosy barb.

Characteristics of Embryogenesis

In this study, all eggs were collected from a single batch. It has been observed that in fertilized eggs, blastodisc formation begins within the first 2 hours and the early morula stage occurs in the 3rd hour. It was determined that the formation of the 16-somite stage started at the 12th hour, and the 24-somite phase started at the end of the 24th hour. Within the 27th hour, the appearance of the embryo became completely clear, and at the earliest 28th hour, decapsulation of the eggs was observed. Figure 4 shows microscopic images of the stages of embryological development from fertilized egg to hatching stage and Table 1 details each stage of embryogenesis.

Characteristics of Larval Development

The total length of newly hatched larvae with yolk sacs was 3245 ± 23 µm. It was observed that the larvae fed endogenously with the vitellus until the 4th day. It was determined that the initial mouth gap size of the larvae was small (304 ± 33 µm). Therefore, the first feeding should be made with small particles such as rotifer or egg yolk, and it was observed that the mouth size became suitable for feeding with Artemia from the 8th day. The post-larval stage started on the 17th day and the transition to artificial powder feed occurred was observed.

Figures 5 and 6 show microscopic images of the sampled larvae. Morphometric values such as the pre-larvae's first total length, the yolk sac's diameter, and the first mouth gap size were measured throughout the development from the first hatching to the post-larval stage (Table 2).

Water quality during egg hatching and larval development was measured, with pH at 6.93 ± 0.4, EC at 49.1 ± 07 µS/cm, salinity at 0.04 ± 0.01 mg/L, DO at 7.34 ± 0.12 mg/L, and temperature at 26.30 ± 1.20°C.

It was determined that the larvae completely absorbed the yolk sac at the 68-71th hour, and mouth opening occurred at 4 DAH. At the opening of the mouth and anus, the yolk was entirely or almost exhausted. Larvae with newly opened mouth gaps are unsuitable for feeding on newly hatched *Artemia*, so crushed egg yolk was the first food. Since egg yolk was observed in the stomach and digestive tract of fed larvae, they started exogenous feeding at the end of the 4 DAH. In addition, it was observed that the larvae were generally motionless on the aquarium floor until 4 DAH, and they started to swim freely after exogenous feeding. The larvae used the entire water column from the 5 DAH. At the end of the 8 DAH, the last segments of the notochord have started to turn upwards, and the caudal fin is turned down, as in the veil tail appearance. The larvae reached the mouth gap

size (412 ± 16µm) that could consume *Artemia* at the end of the 8 DAH, and they fed on brine shrimp from the 9th day. It was observed that the swim bladder became constricted from the 11 DAH and took an utterly two-lobed appearance on the 13th day. The important ontogenic stages and feeding pattern observed during larval development are schematized in Figure 7.

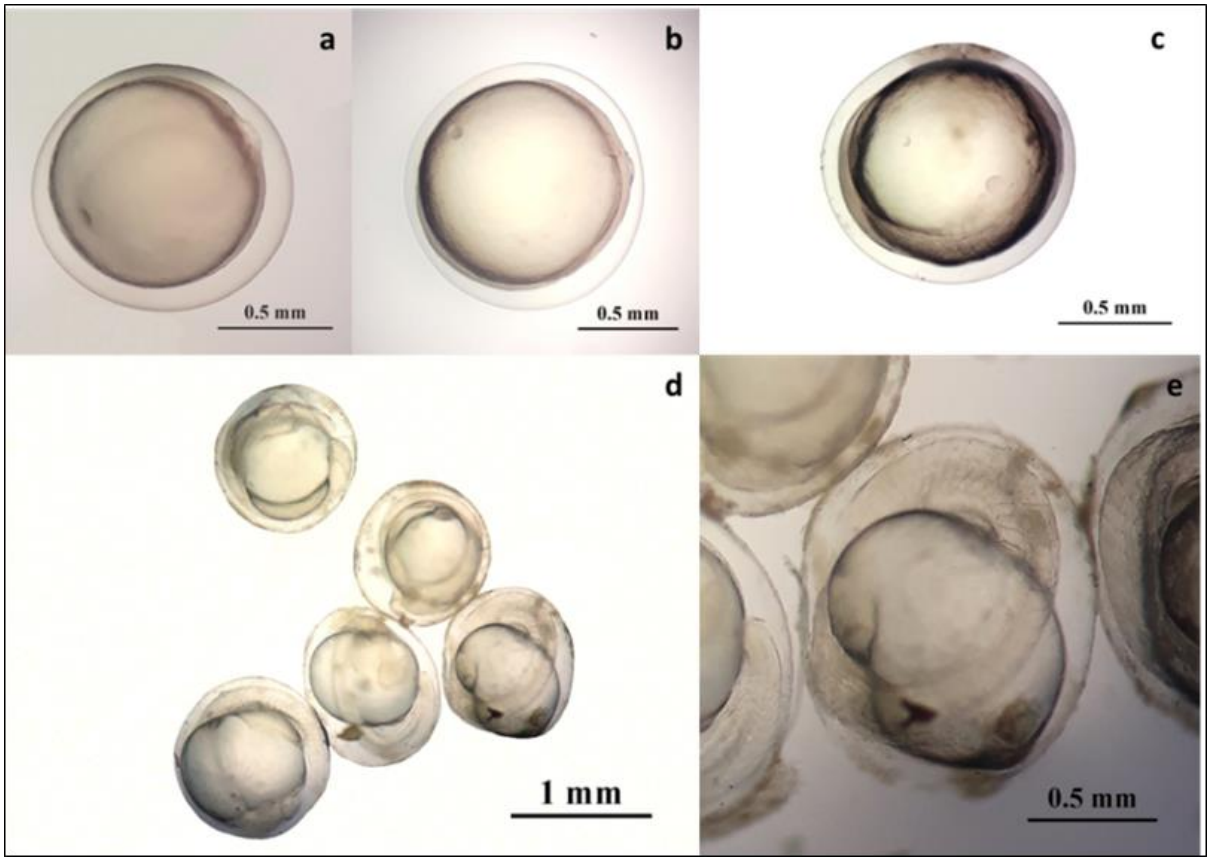

Figure 4. Eggs developmental stages from newly laid eggs to 27th hour. (a: 2nd hour, b: 6th hour, c: 12th hour, d: 24th hour, e: 27th hour).

Table 1. Descriptions of the embryonic development stages (VD: Vitellus diameter, CD: Chorion diameter, ED: Egg diameter. Eggs given embryonic development stages were incubated at 26.3°C and hatched in 27 hours).

Figures	Descriptions/Measurements
a	2nd hour, VD 712 µm, multi-cell (> 64) formation-early morula stage, CD 891 µm, PS 98.7 µm
b	6th hour, 2 somite formation, VD 1067 µm, CD 1213 µm, PS 87.2 µm
c	12th hour, embryo formation, VD 1127 µm, CD 1232 µm, PS 53.1 µm
d	24th hour, embryo formation, ED_1 1608 µm, ED_2 1227 µm, ED_3 1187 µm, ED_4 1197 µm, ED_5 1166 µm
e	27th hour, embryo formation ED 1247 µm

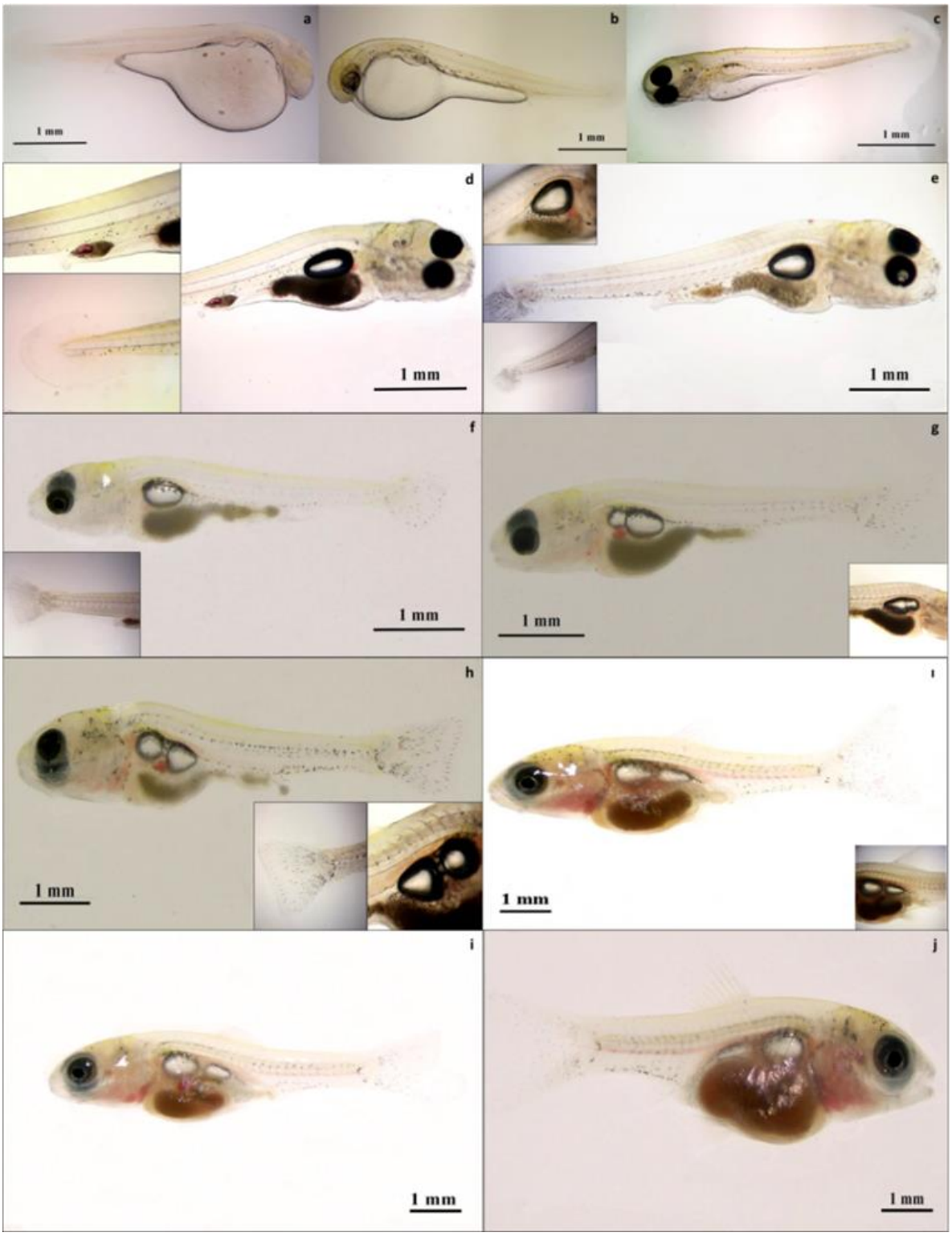

Figure 5. Developmental stages from newly hatched prelarvae to 25 days after hatching (DAH). (a: 1st hour, b: 24th hour, c: 3 DAH, d: 4 DAH, e: 8 DAH, f: 9 DAH, g: 11 DAH, h: 13 DAH, ı: 18 DAH, i: 21 DAH, j: 25 DAH, Scale bar: 1 mm).

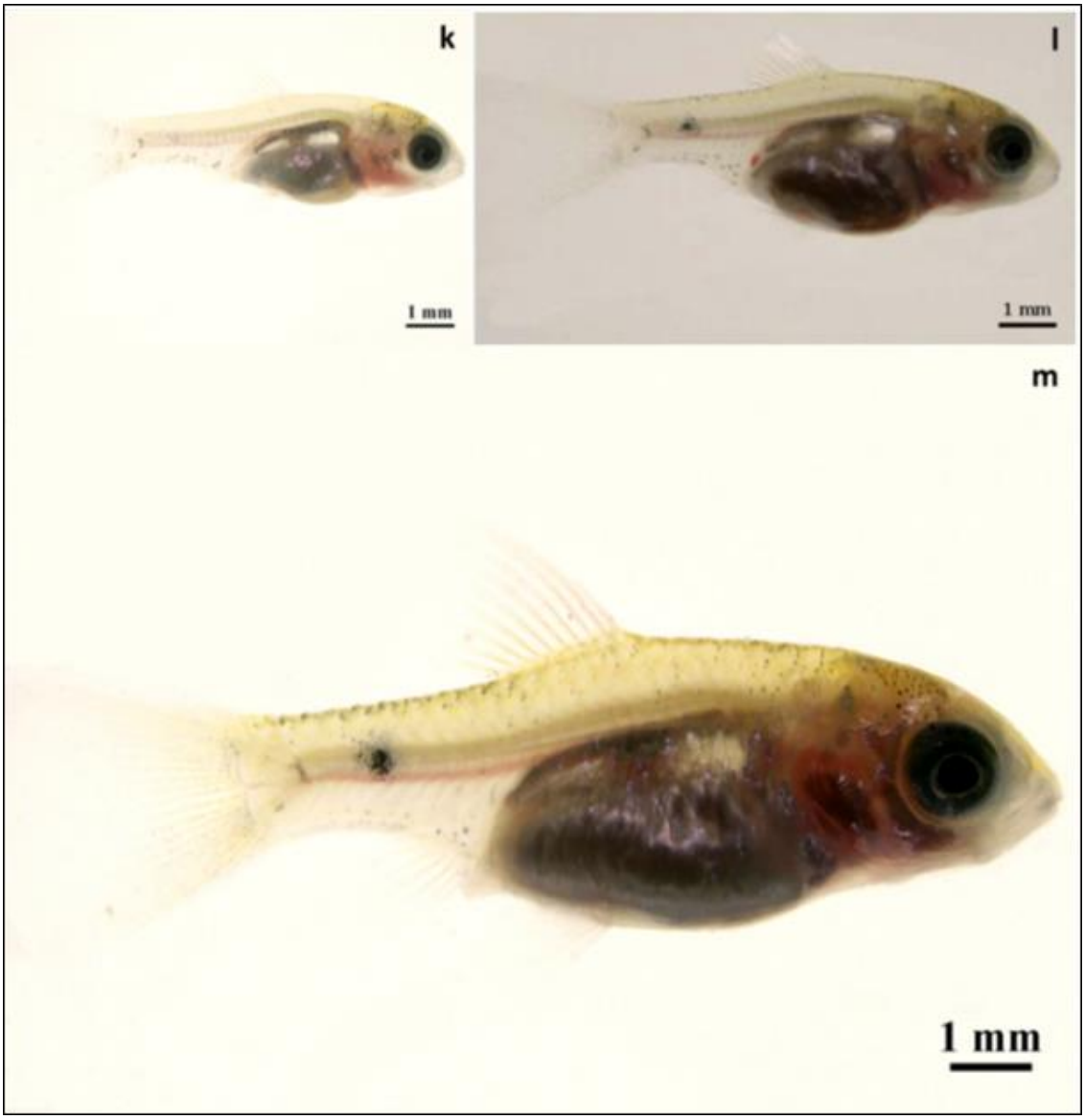

Figure 6. Developmental stages of post-larvae from 29 to 50 DAH. (k: 29 DAH, l: 35 DAH, m: 50 DAH, Scale bar: 1 mm).

Table 2. Descriptions of images about morphometric measurements (DAH: days after hatching, TL: Total Length, YD: Yolk Sac Diameter, MS: Mouth Gap Size. SA: Short axis, LA: Long axis. The larvae in the pictures were kept at 25.4-26.3°C throughout the sampling).

Figures	Descriptions	Figures	Descriptions
a	1th hour, TL 3842 µm, YD: SA 1264 µm, LA 2346 µm	**h**	13 DAH, TL 6400 µm, MS 598 µm
b	24th hour, TL 3977 µm, YD: SA 970 µm, LA 2513 µm	**ı**	18 DAH, TL 9205 µm, MS 801 µm
c	3 DAH, TL 3999 µm, YD: LA 1153 µm, SA 206 µm	**i**	21 DAH, TL 9227 µm
d	4 DAH, TL 4826 µm, MS 279 µm	**j**	25 DAH, TL 9338 µm
e	8 DAH, TL 5673 µm, MS 394 µm	**k**	29 DAH, TL 9570 µm
f	9 DAH, TL 5734 µm, MS 411 µm	**l**	35 DAH, TL 9896 µm

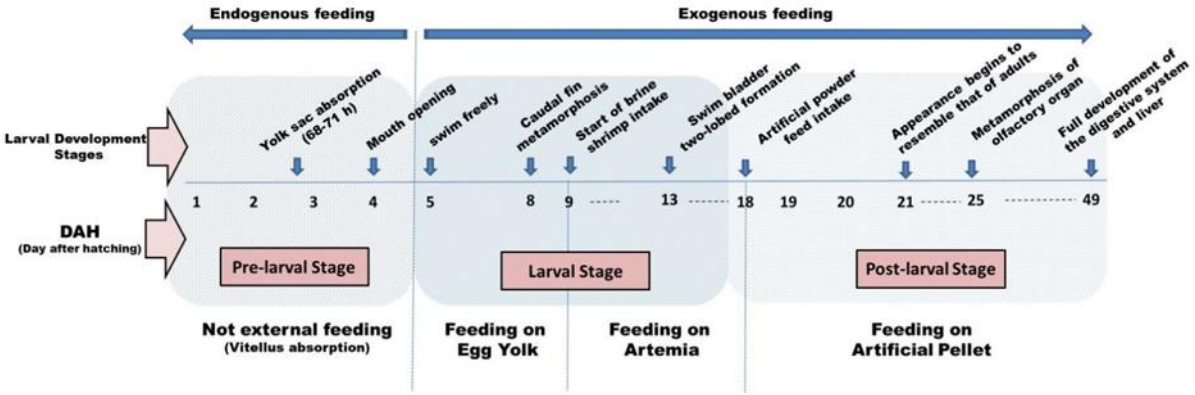

Figure 7. Significant ontogenic changes that occur from the pre-larval stage to the post-larval stage.

Larvae started taking particulate pellets at the end of the 18 DAH. Species-specific black dot pigmentation on both sides of the caudal peduncle was first observed on the 21 DAH. In line with the data obtained, the larvae's early-stage measurement and feeding procedures are summarized in Table 3.

When the larval development stages were evaluated histologically, the digestive tract was not differentiated and segmented on the 5th day, and the liver and the other organs became prominent at the beginning of the 6th day.

The swim bladder became constricted from the 7 DAH, and the digestive tract was differentiated into three regions: the esophagus, stomach, and intestine, which were separated from the 8 DAH. Furthermore, the esophagus became distinguishable at the end of the 8th day. From the 25 DAH onwards, it was observed that the metamorphosis of all nasal system structures was completed. Primarily, this system structure, such as the olfactory organ and olfactory bulb, became fully evident for the first time on the 25th - 27th day. The digestive tract, liver and heart were developed entirely on the 49th – 52th DAH (Figure 8).

The early stage growth formula of rosy barb calculated with the exponential relationship model is $y = 3.8346e^{0.0313x}$ ($R^2 = 0.9112$, n=140). Growth rates of body characters according to total length were estimated according to the allometric equation ($Y = a \times (W^b)$) (Figure 9). From the prelarval stage, HL and ED showed isometric growth, while PrAM and PoAM parameters showed positive allometric growth. From the postlarval stage, PrAM and PoAM showed negative allometric growth.

Table 3. Early larval morphometric measurements and feeding protocol up to postlarval stage (DAH: days after hatching, TL: Total Length, MS: Mouth Gap Size).

DAH	Morphological Measurements/Descriptions (n:120)	Feeding Procedures
1	TL min 3201, max 3271, 3245±23 µm	*not exogenous feeding*
2	TL min 3303, max 3324, 3317±7 µm	*not exogenous feeding*
3	The yolk sac absorption (Almost all of the larvae)	*not exogenous feeding*
4	Mouth opening, MS 304±33 µm	*Egg yolk*
5	MS 369±14 µm, TL min 4373, max 4411, 4379±11 µm	*Egg yolk*
7	MS 401±13µm, TL min 4703, max 4891, 4806±53 µm	*Egg yolk*
9	MS 423±11 µm, TL min 5656, max 5701, 5679±15 µm	*Egg yolk +Artemia*
18	MS 793±19 µm, TL min 7231, max 7506, 7368±95 µm	*Artemia +Particulate pellets*
20	MS 803±21 µm, TL min 8722, max 8889, 8814±53 µm	*Particulate pellets*

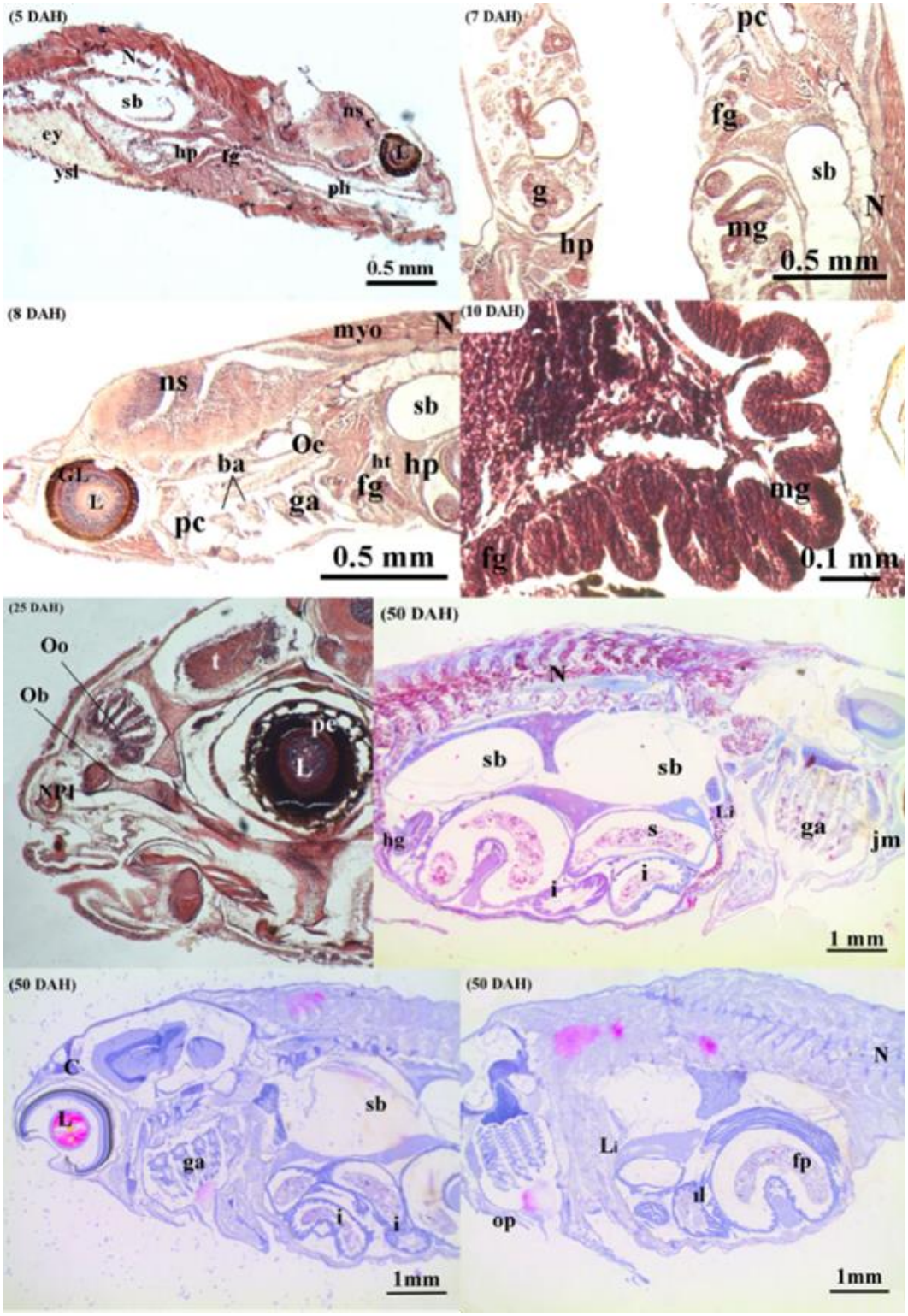

Figure 8. Histological images of larval development stages. (*sb: swim bladder, N: notochord, fg: foregut, hg: hindgut, mg: midgut, hp: hepatopancreas, pc: pharyngeal cavity, ga: gill arch, ey: egg yolk, ns: nervous system, myo: myotome, pe: pigment, ysl: yolk syncytial layer, L: lens, C: cartilages, ht: heart, g: ganglionic layer, ba: branchial arches, NPI: nasal pit, fp: food particles, Li: liver, ıl: intestinal loops, op: operculum, i: intestine, Oo: olfactory organ, Ob: olfactory bulb, t: telencephalon, jm: jaw muscle, s: stomach, GL: ganglionic layer, ba: branchial arches, Oe: esophagus, ph: pharynx).

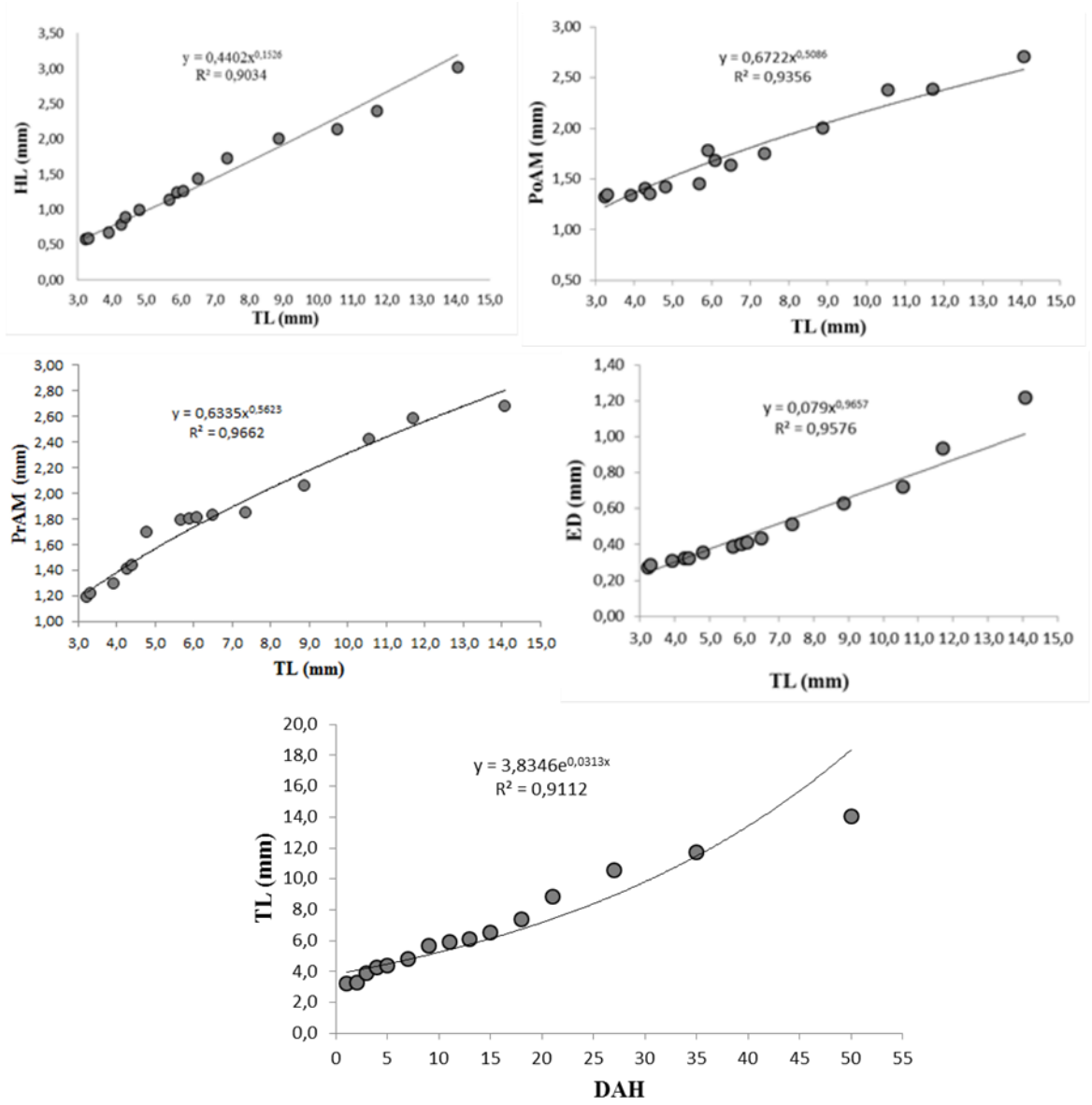

Figure 9. Allometric development curves of morphometric characters during the larval developmental stage and their relationship graphs according to total length (TL: Total Length, DAH: days after hatching).

DISCUSSION

Breeding and Egg Characteristics

In ornamental fish production, some manipulations in a positive way on water quality parameters (EC, temperature and pH that will positively trigger some reproductions are required in line with the requirements of the fish species. There are many studies on breeding ornamental fishes and larval development to determine the optimum water quality (Sezen and Olmez 2012; Sepil et al. 2022). In Amazonian discus species (*Sypmhysodon* spp.), EC at 50-200 µS/cm, pH at 3.9 - 5.7, and temperature at 28.6 - 30.2°C were found to have a reproductive-triggering effect (Çelik et al. 2008). The pH value is between 5.5 - 6.5, the EC value is in the range of 28 - 30 µS/cm and the using humic acid (0.04%) is essential in the breeding of Neon Tetra *Paracheirodon innesi* (Kucharczyk et al. 2010). Optimum breeding values for Tiger Barb *Puntius tetrazona*, which has very similar characteristics with rosy barb, water temperature, pH, and dissolved oxygen, have been reported at 28 ± 0.7ºC,

7.2 ± 0.12 and 6.4 ± 0.34 mg/L, respectively (Abolhasani et al. 2014). When rosy barb is evaluated (26.3 ± 0.4°C, EC 49.1 ± 3.2 µS/cm and pH 6.90 ± 0.10), it is seen that it does not need different special conditions for reproduction with very low pH and EC values, and it lays eggs under conditions similar to other barb species.

Embryonic and Larval Ontogeny

Following the absorption of the yolk sac, mouth opening occurs and exogenous feeding begins in larvae. When the larval metamorphosis of *P. conchonius* is examined, it is seen that the yolk sac is absorbed in a relatively short time (68th - 71st hour), and exogenous feeding begins (4 DAH) in the newly hatched pre-larva compared to other ornamental fish species. In the black neon tetra *Hyphessobrycon herbertaxelrodi*, the yolk sac is completely consumed on the 5th DAH and the average total length of the larva is around 3.70 mm (Çelik, et al. 2011), in the goldfish *Carassius* sp., the yolk sac is completely absorbed at the 81st hour (Savaş and Timur 2006), in the jaguar cichlid *Parachromis managuensis*) the vitellus absorption that occurs on the 4th day (Arık 2013) has been reported. The table in which some aquarium fish species critical early development stages are schematized and compared to the rosy barb is given in Figure 10.

The larva's newly opening mouth gap size (304 ± 33 µm, n: 30) is quite large compared to other species. This situation allows the egg yolk or rotifer-based feeding period to be short and the larvae to get *Artemia* in a shorter time (9 - 10 DAH). These features are considered important in the species' low early larval losses. However, the fact that it can be fed directly with *Artemia* after consuming egg yolk for only a short time makes the maintenance and production of the fish easy for amateur hobbyists.

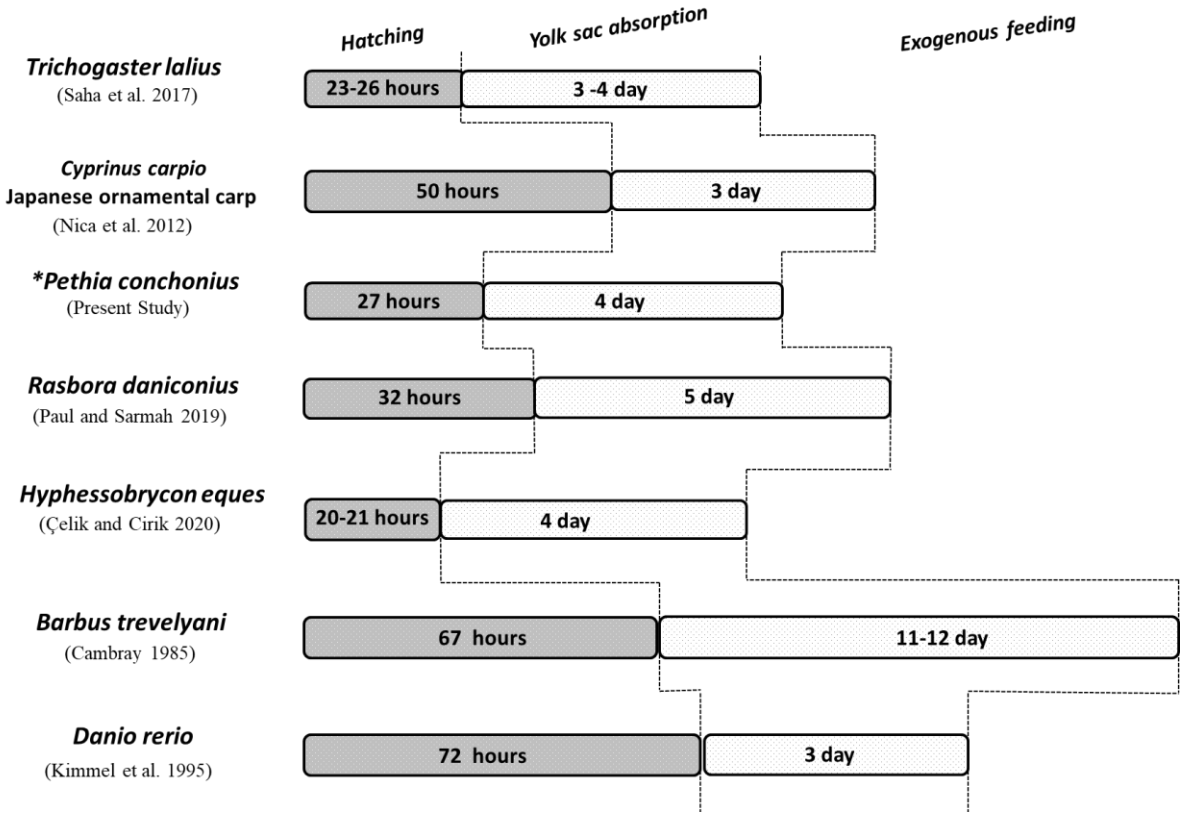

Figure 10. Egg hatching, endogenous and exogenous feeding periods in some important aquarium fish species.

In addition to microscopic examination of the development of fish larvae, there are histological methods to determine the development of tissues and organs in ontogeny studies. There are studies in which microscopic examinations are supported by histological methods in revealing larval ontogeny (Sarasquete et al. 1995; Monsefi et al. 2010; Santos et al. 2016). In the work, the stages of various tissue and

organ differentiation that cannot be visualized microscopically were determined in histological sections. The gill arch and pharyngeal cavity became prominent (5 DAH), the digestive tract differentiated into anterior and posterior intestines, and the hepato-pancreas became evident (7 DAH). The fact that this period is short ensures the formation of a functional stomach in a short time. The transition of rosy barb larvae from live food to artificial powder food takes place in a shorter time than many other ornamental fish larvae, and thus, the feeding time with live food appears to be shorter. This is so crucial in terms of low live food costs and larval losses in the culture of the species.

Allometric Growth Measurements

Allometric growth models are presented in this study, which reveal the allometric growth parameters in the larval stage of the rosy barb. This method is widely used to analyze the growth relationships in the early larval stages of fishes (Peña and Dumas 2009; Çelik et al. 2011). Allometric growth of fish groups in their larval stages was studied based on family and species (Osse and Van den Boogaart 2004). There is no scientific literature on the growth of many ornamental fish of commercial importance. It is thought that the data on the larval development of the rosy barb, which is a commercially essential and popular aquarium fish, will contribute to the knowledge.

Ontogeny and Feeding Relationship

Histological methods have been used for monitoring the development of tissues and systems in the early larval stages of fishes, significantly to determine the structural metamorphosis of the stomach and entire digestive tract and turn into a functional stomach (Onal et al. 2008; Ramezani Fard et al. 2011). Limited enzyme activity and an undeveloped digestive system in the early life stage of fish prevent powder feed consumption (Onal et al. 2008). This situation necessitates using various zooplankton species (*Artemia* sp., *Brachionus plicatilis* and infusoria) in this stage. The mouth gap size of the larvae determines which of these live foods will be used in the first feeding. Especially live foods are the highest cost in larval feeding in commercial fish culture (Onal 2006; Sepil and Şen 2024). Therefore, determining how long zooplankton feeding will be followed by powder artificial feeds depending on the mouth gap size and digestive system. So, these physiological and morphometric developments are closely related to the effective use of microscopic and histological methods in larvae ontogeny studies.

Generally, rosy barbs are thought to be among the species with high commercial returns that hobbyists easily prefer in mixed-species aquariums, especially due to their veil-tails, attractive colors, and ease of maintenance. In this respect, it is very important to know the larval development stages of this species and other ornamental fish species well for the aquarium fishing industry.

FUNDING

This study was conducted using university facilities without any institutional financial support.

ETHICAL CONSIDERATIONS

The study was carried out following experimental animal ethical rules, grand number 2021/11-10.

DECLARATION OF COMPETING INTEREST

The authors declare that there is no competing interests to any authors.

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ROLE OF AUTHORS: *AS – carried out the experimental design of the aquariums used in the trial, the feeding of the fish and the egg collection, conducted water quality monitoring throughout the trial, and performed daily measurements and allometric calculations of the larvae. ZAÇ and BEA – performed the histological processing and evaluated the sections. ZAÇ, BEA and ARO – performed immunohistochemical stainings and cell counts. AS and ARO – wrote and revised the manuscript. The final version of the text was read and approved by all authors.*

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