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# Preliminary studies on morphology and digestive tract development of tomato clownfish, *Amphiprion frenatus* under captive condition

[1,2]Dedi F. Putra, [3]Ambok B. Abol-Munafi, [2,4]Zainal A. Muchlisin, [1]Jiann-Chu Chen

**[1]Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwan ROC; [2]Department of Aquaculture, Syiah Kuala University, Banda Aceh, Indonesia; [3]Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Terengganu, Malaysia; [4]Tsunami and Disaster Mitigation Research Center, Syiah Kuala University, Banda Aceh, Indonesia.**

**Abstract.** The study investigated on the growth, morphology and digestive tract development of *Amphiprion frenatus* larvae under captivity condition. A total of 15 larvae were sampled from 1 day after hatching (DAH) to 20 DAH for measurement of total length (TL), head depth (HD), eye diameter (ED), body depth (BD), head length (HL) and standard length (SL). For data analysis, total length (TL) was used as variable with respect to other morphometric characters to plot relative growth curve. The relative growth equation of SL, ED, BD and HD, HL was established by using Regression. A total of 15 larvae were sampled from 1 DAH to 17 DAH for histology procedure. Result showed that *A. frenatus* hatched at the advance stage with developed and functional eye, mouth and fins. Pectoral fin forms at 3 DAH (5.229 ± 0.17 TL). Notochord flexion occurred at 5 DAH (5.675 ± 0.07 TL). The complement of the notochord was characterized by the orientation of the caudal fin rays at 4 DAH (5.399 ± 0.08 TL). The reddish coloration appeared on 17 DAH (9.7695 ± 0.22 TL). At 20 DAH (13.31 ± 0.83 TL), two white bends were formed and bordered by melanophores along the bend. The total length (TL) has significant relationship with standard length, head length, head depth, body depth and eye diameter. Histology of digestive tract of *A. frenatus* larvae at 24 hours after hatching showed alimentary tract is evident, with gut and liver differentiated. At 2 days after hatching, a well developed alimentary tract is evident, with distinct stomach, midgut and hindgut. Liver and pancreas are differentiated. On the 5 DAH (5.675 ± 0.07 TL) mouth cavities are well developed. The mouth and pharynx were lined with cubic epithelial cells of irregular shape. It could be concluded that after 8 days incubation, *A. frenatus* hatched at the advance stage with developed and functional eye, mouth and fins. Relative growth concluded that the total length (TL) has significant relationship with SL, HL, HD, BD and ED. Histology of digestive tract of *A. frenatus* larvae showed that the *A. frenatus* larvae have a straight tube closed at the mouth and histological differentiated along its length at newly hatching. It was found also several functional organs such as liver, midgut and stomach at 1 DAH. It was indicated with the appearance of developed and functional digestive tract at first day hatching larvae. Therefore, live foods such as rotifer are suitable to be fed at early larvae after hatching.

**Key Words:** *Amphirion frenatus*, fish larvae, morphology, growth, histology.



**Corresponding Author:** Dedi F. Putra, df_putra@yahoo.com

## Introduction

Ornamental fish farming has become one of the major aquaculture economic activities in Malaysia today. Unknown to most, it started as a backyard industry in the early 1960s in the northern state of Penang. Most of the cultivated species in Malaysia originate from America, Africa, and other Asian countries (Lozinsky 2011). Fries of indigenous species are collected from the wild during seasons all year round, and raised to marketable size (Leong 2006). Successful experiments on the breeding of ornamental marine fish species are limited. This is due to the fact that rearing of tropical marine fishes are difficult compared to freshwater species. Tropical marine species are beautiful as some marine species, such as members of Pomacentridae, are important in the world trade for ornamental fish (Wilkerson 2001) and a popular subject of research (Arvedlund *et al* 2000).

The tomato clownfish or, after its scientific name, *Amphiprion frenatus* Brevoort, 1856, also known as blackback anemonefish, fire clown, one band anemonefish, or red clown are under Order Perciformes which contain approximately 9293 species (Jung 2006). This is the large order dominated by marine shore fishes.

The early development of fishes is usually regarded as a steady accumulation of small changes to produce an adult phenotype. Marine fish metamorphosis is marked by dramatic changes from larvae to juvenile or young fish. These dramatic changes make rearing marine species a challenge that are not found in freshwater fishes (Sahandi 2011). The transformation from larvae to juvenile can be quite abrupt, which most clownfish larvae reach metamorphosis before 14 days old (Wilkerson 2001).

Larvae morphological characters include larva, fin fold, differentiation of fins, temporary organs, absence of scale cover, non-adult like body proportions and pigmentation development (URHO 2002). Daily growths of morphological development have been major advances in understanding of the early life history of fish larval (Van Snik *et al* 1997). The discovery of daily growth increments in microstructure of otoliths (Green & Fisher 2004) to provide age and growth estimates has revolutionized the interpretations of ontogeny and early life history traits (Fuiman & Higgs 1996).

As embryogenesis and/or larval development of many other model organisms (mouse, newts etc) is well studied (Pall *et al* 2010; Cicek 2011) the literature regarding early stages development in clownfish is rather poor wideworld.

An understanding of normal larval morphology in clownfish species is critical as it may be used to evaluate culture condition of mass production of high quality juveniles. Furthermore, there have no species detailed literature on morphology development. The ability to produce a complete series of the early stage specimen will provide an opportunity to enhance our understanding of the early development of *A. frenatus*.

## Materials and Methods

### Source and larvae rearing

Tomato clownfish, *A. frenatus* larvae was obtained from natural spawning of a wild captured broodstock. Broodstocks were fed daily with pellets containing 50% protein. For stimulating the spawning, cockles with *Spirulina* sp. were used to feed. The larvae were reared at the larvae rearing tanks for 20 days, a period which corresponds to the middle of larval development. Phytoplankton *Nannochloropsis oculata* were added daily to serve as water conditioner and as a direct food to rotifer. Larvae were fed for the first time at 6 hours after hatch with SS – rotifers (*Brachionus plicatilis*) enriched by *Spirulina*. An initial prey concentration was five rotifers per mL. Artificial feed were fed since 15th DAH. From 2nd DAH to 15th DAH larvae were fed by *Artemia* nauplii. The waste material was siphoned out from the bottom of the rearing tank after seven days.

Several parameters such as temperature, pH, dissolved oxygen (DO) and total dissolved solids (TDS) were measured using YSI Horriba water quality measurements. General water quality were maintained at temperature 27.8 – 28.9 °C, salinity at 30 – 32 ppt, DO at 4.9 – 5.45 mg/L, photoperiod at 12L:12D, pH at 7.23 – 8.41 and ammonia at 0.0012 – 0.0024 mg/L.

### Sampling and observation on morphology

A total of 15 larvae were sampled from 0 DAH to 20 DAH throughout the study. Observation and measurement were focused on total length (TL): from the tip of the lower jaw to the posterior margin of caudal fin; standard length (SL): from the tip of the snout to the tip of the notochord for larval stage and to the base of the caudal fin (posterior margin of the hypural plate) for the post flexion specimen; pre-anal length (PL): from the tip of the snout to the anus; head depth (HD); body depth (BD): from the anus to upper limit of the mymore excluding dorsal fin hold area; upper jaw length (UJL) and eye diameter (ED) (Figure 1). Metamorphosis with any appearances of abnormality was excluded to analysis. Sampling was performed early in the morning before feeding. Samples of larvae were anaesthetized in 100 ppm 2-phenoxyethanol solution and morphometric characteristics were measured with Nikon measurement microscope. For morphological visualization, Compound and dissecting microscope equipped with Camera Lucida was used to draw the larvae. After measurement, the larvae were preserved in 10% buffer formalin and kept at 20°C for storage.

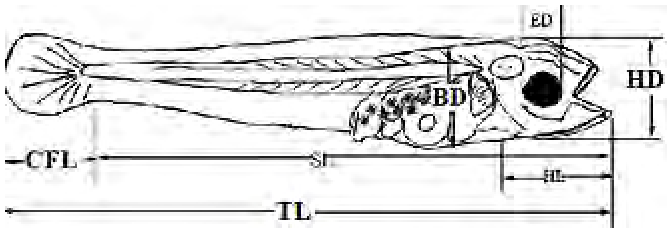


**Figure 1.** Morphological characters measured in tomato clownfish larvae. CFL, caudal fin length; HD, head depth; HL, head length; SL, standard length; BD, body depth; ED, eye diameter; TL, total length (Allen 1974; Önsoy *et al* 2011).

### Growth rate analysis

A total of 300 of fish larvae were sampled to determine the growth performance at the end of experiment. Nikon profile projector Model V-16A was used to measure the total length of larvae. Mean growth rate was calculated as the daily length increase (mm.day-1) using the formula of Ricker (1979).

### Sampling on development of digestive tract

Sampling and histological analysis was used based on Haematoxylin-eosin (Drury & Wallington 1967) and Kiernan (1990). Fifteen normal larvae were taken daily from hatching 0 DAH to 7 DAH. Sampling was conducted every day before feeding larvae in the morning.

### Data analysis

For data analysis, we used of TL as an independent variable with respect to other morphometric characters to plot relative growth curve. The relative growth equation of SL, ED, BD and HD, HL was established by using Regression. The following Relative growth equation was used: Y = ax + b; where, Y = measured characters; a = intercept and b = slope or growth coefficient (Fuiman 1983; Gisbert *et al* 2002).

## Results and Discussion

### Morphological development

The early development of fishes is usually regarded as a steady accumulation of small changes to produce an adult phenotype. The transformation from larvae to juvenile can be quite abrupt, and most clownfish larvae reach metamorphosis before 14 days old (Wilkerson 2001) (Figure 2). For newly hatched *A. frenatus* larvae, the major morphological organs already formed. It is indicated that the larvae hatched at the advance stage. URHO (2002) highlighted that, larvae morphological characters include larva; fin fold, differentiation of fins, temporary organs, absence of scale cover, non-adult like body proportions and pigmentation

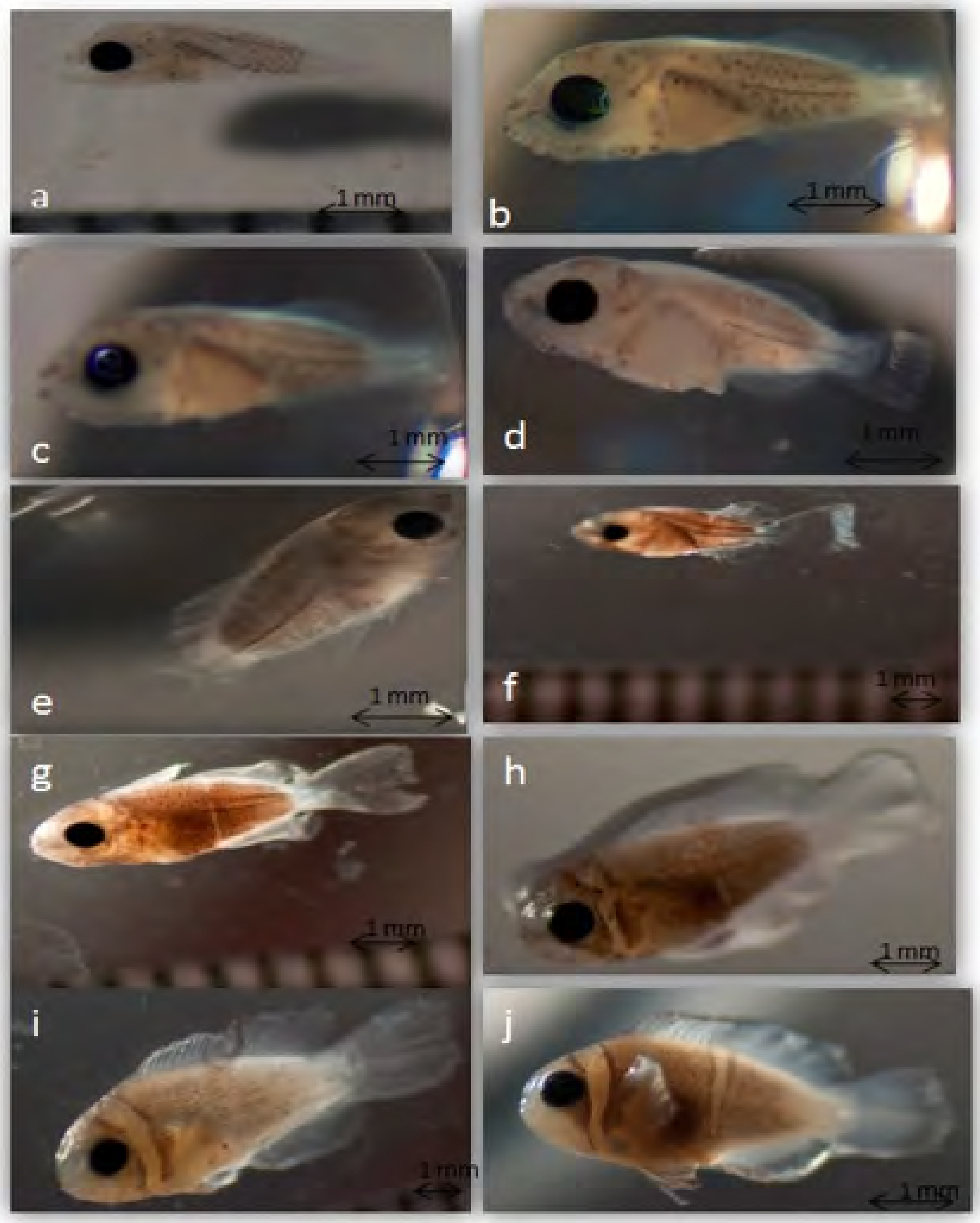


**Figure 2.** Larval and post larval tomato clownfish (a) Post larval stage (1 DAH 4.61 ± 0.11 mm); (b) 2 DAH TL 4.768 ± 0.09 mm, forming a curve at caudal part; (c) Pelvic fin performed at 3 DAH (5.229 ± 0.17 mm TL); (d) The complement of the notochord flexion at 4 days post hatching TL 5.399 ± 0.08 mm;(e) The completion of notochord flexion occurred at 5 DAH; (f) The melanophores were replaced by chromatophores;(g) White band in the middle body region formed at 10 DAH (8.15 ± 0.3 mm TL);(h) At 13 DAH (8.79 ± 0.54 mm TL), the white bands on the posterior of head are visible; (i) Post juvenile stage 17 DAH (9.7695 ± 0.22 mm TL), the reddish color formed near to pelvic fin; (j) At 20 DAH larvae (13.31 ± 0.83 mm TL) two white bands are formed.

development. The metamorphosis phase occurred on 4th DAH which indicated the transformation period from the pre-flexion to post-flexion stage. *Amphiprion* species have a continuous dorsal fin with 9 - 14 spines, 11 - 18 soft rays and anal fin with two spines. *Amphiprion* species can grow to a maximum length of about 35 cm, but adult *Amphiprion ocellaris* Cuvier, 1830 grow to slightly over three inches (80 mm) in standard length (Wilkerson 2001). Compared to the statement, *A. frenatus* has the similar number of fin because it belongs to genus Amphiprion.

The initial of the upward bending of the notochord and the completion of caudal fin rays formation of *A. frenatus* occurred at 4 DAH. It was indicated the transformation period from the pre-flexion to post-flexion stage. The yolk of *A. frenatus* larvae had been completely exhausted at 1 DAH with 4 mm in TL. This period was defined as the period of progression from the larval to post-larval stage. Progression period from the pre larval to post-larval stage of Ryukyu-ayu (*Plecoglossus altivelis ryukyuensis* Nishida, 1988) larval was recorded at the size 7 - 8 mm TL (Tachihara & Kawaguchi 2003). According to Mihelakakis *et al* (2002), size of newly hatched larvae, time of hatching and rearing conditions can influence the commencement of notochord flexion which is the species-specific dependent.

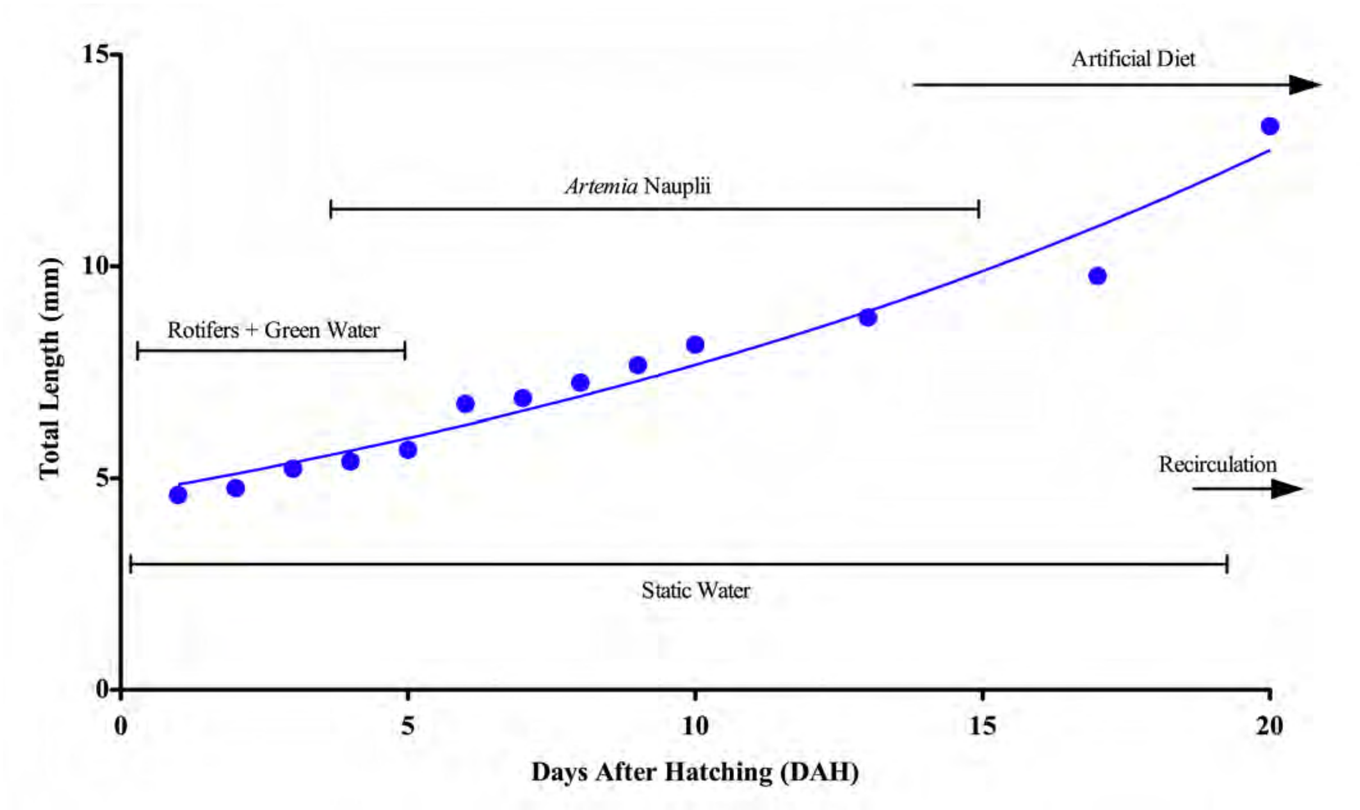


**Figure 3.** The growth pattern and feeding regime of *Amphiprion frenatus* larvae until 20 DAH.

In previous study reported by McFarlane *et al* (2000), the first metamorphosis marked by rapid growth and development of structures, particularly in body depth, body length, jaw development and improvement of locomotion ability, was classified as the pre-juvenile stage, associated with the flexion classification. Coloration and full complement of fins occurrence was specific to the post-juvenile stage. Based on this definition, the pre-juvenile stage of *A. frenatus* larvae was detected at 13 DAH (Figure 2).

*A. frenatus* larvae in this study had a similar sequence of fin formation as shown in other teleosts species during the early life stage with primary formation of pectoral fin, followed by caudal fin, soft-ray dorsal fin, anal fin and dorsal spines during post-larvae and pre-juvenile stage. It has been shown that incipient rays of the pectoral and caudal fins for blenny species begin to form before notochord flexion and segment during flexion (Ditty *et al* 2005).

The post-larval stage of *A. frenatus* started to transform forked tail into rounded tail at 15 DAH during pre-juvenile stage. Mihelakakis *et al* (2002) reported that swim bladder inflexion and establishment of hydrostatic regulation of generally physoclistous fishes larval occurred at onset of external feeding and coincided with complete yolk-exhaustion. Fully pigmented and functional eye of *A. frenatus* grew symmetrically on both sides of the head at hatching. The pigmented eyes allowed the larvae to swim and feed immediately after hatching. The simple retina of the early larval stage of bony fish containing only green sensitivity rods and cones enable them to detect light intensity, signals of night or day times and the contrast between potential predator and prey (Kasumyam & Kazhlayev 1993; Gisbert 1999).

However, the results obtained in laboratory-reared specimens might be slightly different from those reported in wild population. These differences might be influenced by different environmental parameters that regulate their development. Temperature is the most important environmental factor for metamorphosis of fishes during early life development (Mihelakakis *et al* 2002).

**Growth rate and relative growth**

Blaxter *et al* (1983) stated that the body size at hatching is the key for survival as the larger size is advantageous to survive. Bagarinao (1986) highlighted that many species, especially with small pelagic eggs resulting in larvae of small body size at hatching, increase in size rapidly during the first several hours after hatching and show relatively stable growth thereafter. Compared to this study, the *A. frenatus* larvae mean growth rate was 0.435 mm.day-1, where the mean total length at hatching day was 4.61 ± 0.11 mm and grew to 13.31 ± 0.9 mm by the end of the project (20 DAH) (Figure 3).

As for feeding regime of *A. frenatus*, rotifer and green water were initiated as live food for 1 DAH larvae until 5 DAH. *Artemia* nauplii also fed started from 3 DAH until 15 DAH larvae. The static water was used from first hatching to 20 DAH. Artificial diet such Love Larva fed from 15 DAH forward. The artificial diet gradually changed based on the size of larvae. Grower larvae need bigger size of artificial diet. The establishment of this feeding regime was introduced by Bloch (1997) (Figure 3).

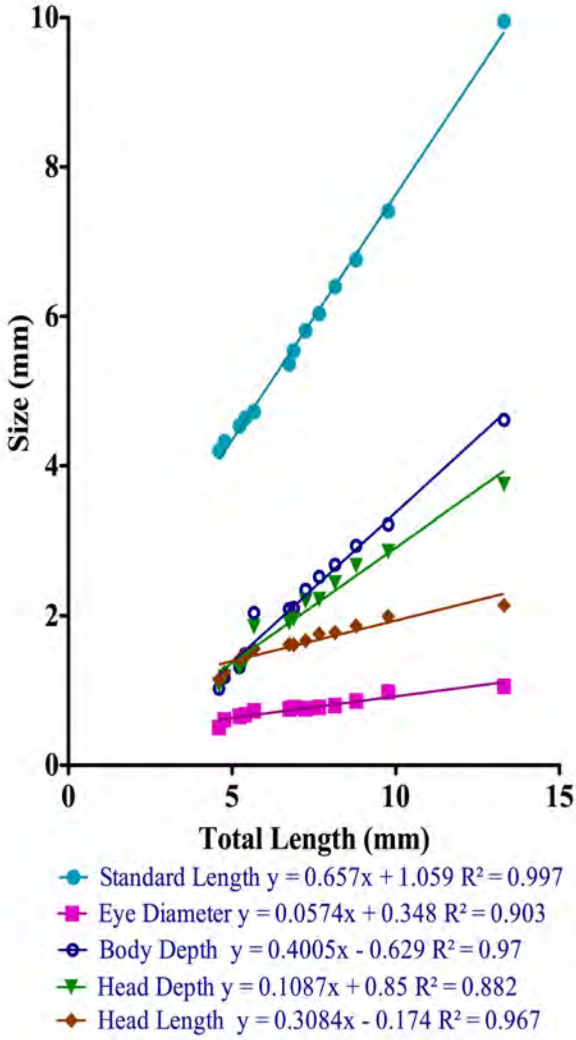


**Figure 4.** Graph showing the significant relationship among total length to standard length, eye diameter, body depth, head depth, and head length with regression equation respectively

The regression equation among TL and SL, HL, BD, HD and ED of *A. frenatus* larvae showed a strong relationship among those morphometric values (Figure 4). Allometric growth occurs when some part of an animal's body grows at a different rate in relation to a reference dimension, generating changes in body proportions. Some changes are abrupt, marking crucial ontogenetic stages, such as prepuberal or puberal molting (Hartnoll 1978).

**Histological development of A. frenatus larvae**

An *A. frenatus* larva has a straight tube closed at the mouth and histological differentiated along its length at newly hatching. It was also found several functional organs such as liver, midgut and stomach at 1 DAH (Figure 5). Development contuined at a remarkable rate (Figure 5 & 6).

Kolkovski (2001) summarized the significant transformation in the digestive system during the ontogenesis of fish larvae. He stated that the digestive tract is a straight tube closed at the mouth and histological differentiated along its length at hatching. It remains quite unchanged from mouth opening until the

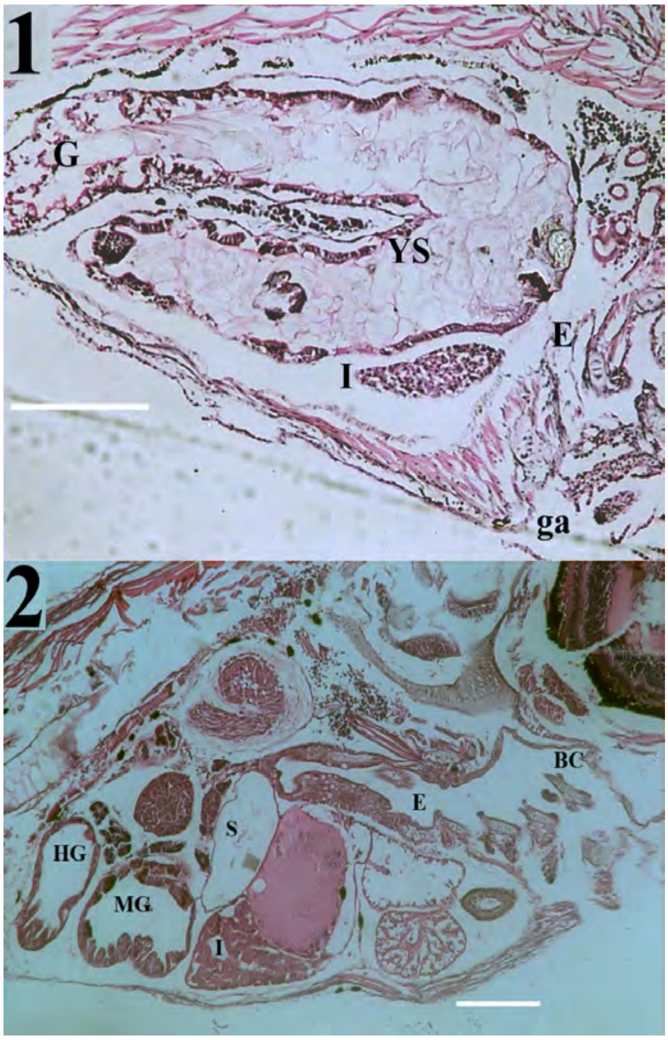


**Figure 5.** Microsection of a tomato clownfish, *Amphiprion frenatus* larvae (1) 2 DAH; a well alimentary tract is evident, with distinct stomach, midgut and hindgut. Liver and pancreas are differentiated. E, esophagus; HG, hindgut; l, liver; MG, midgut; S, stomach. (2) 24 hours after hatching; alimentary tract with gut and liver are differentiated. E, esophagus; ga, gill arches; l, liver. Scale bar: 20 µm

completion of yolk absorption and then becomes segmented into a buccopharinx, foregut, midgut and hindgut. According to Faulk *et al* (2007), the presence of a primordial stomach has commonly been associated with an inability to efficiently rear marine fish larvae on compound diets.

The combination of enhanced swimming capabilities, developing jaw and mouth structures suggest the more diverse and large prey items could be captured and digested to enhance bioenergetics, growth and survival (Kockeis *et al* 2001). Based on this study, the mouth opening on 5th DAH showed the complete and functional buccal cavity, esophagus and pharynx. Therefore, they can digest to enhance bioenergetics, growth and survival at that stage.

## Conclusions

It was concluded that after eight days incubation, *A. frenatus* hatched at the advance stage with well developed and functional eye, mouth and fins. Pectoral fin forms at 3 DAH. Notochord flexion occurs at 5 DAH. The complement of the notochord

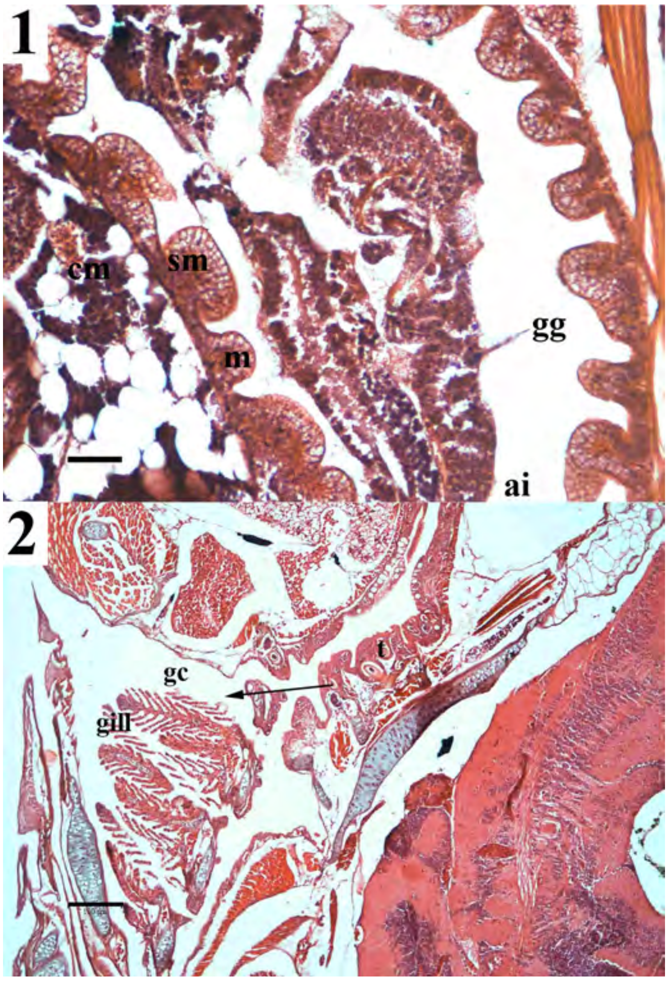


**Figure 6.** Microsection of a tomato clownfish, *Amphiprion frenatus* larvae (1) 14 DAH; sagittal section of the stomach, gastric glads (gg), mucosa (m) and sub mucosa (sm); anterior intenstine (ai), circular muscle layer (cm), liver (L). (2) 17 DAH; goblet cells (gc) and taste buds (t) were visible throughout the epithelial cells of the buccopharyngeal epithelium being more abundant in the anterior of the buccal cavity. Scale bar: 100 µm

was characterized by the orientation of the caudal fin rays at 4 DAH. The reddish coloration appeared on 17th DAH, and at 20 DAH two white bends were formed and bordered with melanophores along the bend.

Relative growth concluded that the total length has significant relationship with standard length, head length, head depth, body depth and eye diameter. Histology of digestive tract of *A. frenatus* larvae showed that the *A. frenatus* larvae have a straight tube closed at the mouth and histological differentiated along its length at newly hatching. It was found also several essential organs such as liver, midgut and stomach at 1 DAH (Figure 5). It was indicated with the appearance of a complete digestive tract at first day hatching larvae (therefore, live foods such as rotifer are suitable to be fed at early larvae after hatching). The further research is useful to investigate the enzymatic process in digestive tract of *A. frenatus* larvae.

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## Authors:

•Dedi Fazriansyah Putra, Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University, Keelung, 202, Taiwan ROC and Department of Aquaculture, Syiah Kuala University, Banda Aceh 23111, Indonesia, e-mail : df_putra@yahoo.com

•Abol Munafi Ambok Bolong, Institute of Tropical Aquaculture, University Malaysia Terengganu, Terengganu Malaysia, e-mail: munafi@umt.edu.my

•Zainal Abidin Muchlisin, Tsunami and Disaster Mitigation Research Center, Syiah Kuala University, Banda Aceh, Indonesia and Department of Aquaculture, Coordinator of Fishery and Marine Sciences, Syiah Kuala University, Banda Aceh 23111, Indonesia, e-mail: muchlisinza@yahoo.com

•Jiann-Chu Chen, Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University, Keelung, 202, Taiwan ROC.


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