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Complete Substitution of Enriched Rotifer by AF-Artemia franciscana and Enhancing Clownfish *Amphiprion bicinctus* Larvae Production via Novel Microalgal Enrichment

Heba S. El-Sayed*, Rania F. Ismail, Hanan M. Khairy, Nevine M. Abou Shabana
National Institute of Oceanography and Fisheries, NIOF, Egypt

*Corresponding Author: hebasaad2222@yahoo.com

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ABSTRACT

Two experiments were performed in this investigation. The first was established to compare the use of enriched rotifers (EROT) against AF *Artemia* (AF Art) for substituting rotifer as start feeding for Clownfish larvae and lasted 7 days. The observations showed a relatively lower capture rate in AF Art treatments on 1st day, although, the intake and assimilation of AFArt nauplii were clearly observed. Accordingly, growth and survival data were non-significant. The second was performed on larvae from 8-21 days after hatching (DAH), evaluating the enrichment impact of EG Art with four microalgae spp., *Chlorella salina*, *Tetraselmis chuii*, and a novel trail (*Rhodomonas salina*) which represented as (CEA, TEA& REA), respectively against *Nannochloroposis oculata* (NEA) as control concerning growth performance, pigmentation, metamorphosis and survival. Additionally, detection of unsaturated fatty acids grouping mainly omega3 ratios in various enriched *Artemia* was performed. Results revealed that the percentage of PUFA, HUFA and n-3 fatty acids were significantly higher ($p<0.01$) in (REA) compared with (TEA) followed by (NEA) & (CEA), respectively. Otherwise, DHA/EPA and EPA/AA ratios were promoted in REA then NEA whereas no values for DHA/EPA were detected in both TEA & CEA treatments due to DHA absence. Larvae group C4 achieved maximum growth of 111.06 ± 0.2mg; on contrary (C2) attained the lowest results. In C4 group; metamorphosis took 9 days, while it extended to 10, 11, and 12 days in C1, C3, and C2, respectively. However, the onset pigmentation of larvae was surpassed in TEA followed by REA within 3-4 days from the beginning of the experiment shorter than NEA. Moreover, the C4 group achieved the highest survival of 80 ± 2.4%, while C2 attained the lowest records. This study authenticated the possibility of using AF-Art as the first feed to facilitate breeding intricacy as also suggested *R. salina* alga enrichment as a prime candidate for improving the Clownfish industry.

INTRODUCTION

Clownfish is one of the most interesting common marine fishes of the genus *Amphiprion* which live symbiotically with sea anemones *Gyrostoma* sp. and *Radianthus* sp. Anemone fishes are in high demand by marine aquarists, and they have become

increasingly popular targets for collection **(Tan and Arai, 2019)**. Both anemone and their Clown fish tenants absolutely rely for their survival on coral, which is facing threats from warming seas, pollution and human intrusion, so they may need to adapt quickly specially, *Amphiprion bicinctus* species that is only inhabiting the Red Sea as reported in the **Manila times Newsettler (2019)**.

Regarding the lack of references, only two studies were published by two colleagues at the Aquaculture Division of NIOF in 2006 and **2008 (Abou Shabana and Helal, 2006; Helal and Abou Shabana, 2008)**. They investigated reproductive performance with monitoring larval and juvenile stages of *A. bicinctus* in laboratory captive conditions.

Currently, the urgent need for aquaculture is being considered as an accountable alternate for the sustainability of colored marine fish trading **(Pouil *et al*., 2020)**. Some authors confirmed that marine fish aquarium in an enclosed system is a way to support marine ornamental fish trade **(Olivotto *et al*., 2017)**. Others referred that the main issue in marine ornamental fish breeding is the larval period for instance many species could produce larvae but these larvae virtually impossible to be maintained under appropriate conditions, including feeding adequacy **(Olivotto *et al*., 2017; Rhyne *et al*., 2017; Callan *et al*., 2018)**.

Knowing the first feeding requirements is a crucial factor influencing successful breeding in the majority of marine fish aquarium species **(Koven *et al*., 1999; Rainuzzo *et al*.,1997; Rønnestad *et al*., 1999, 2003)**. The urgent need for fast growing in economic species, such as *A. bicinctus*, is that the larval diet must match each energetics and nutritional needs. Ornamental fish larvae are usually fed with the rotifer S-type (*Brachionus rotundiformis*), which is a suitable initial diet for the first feeding of the Clownfish larvae at hatching till yolk sac completion has collapse period. So, rotifers were then substituted for the first 7–8 days by larger crustaceans such as *Artemia* **(Divya *et al*., 2011)**. Both Rotifers and *Artemia* have been utilized in larvae culture of marine ornamental fish as principal live feeds to date with successful transition to the juvenile stage **(Baensch and Tamaru, 2009, Madhu *et al*., 2016; Majoris *et al*., 2018)**.

Alternatively, to other live feeds organisms, Rotifers and *Artemia* are favored together by the aquaculture industry because of the relative facility for mass production and their cost-effectiveness. For instance, *Artemia* spp. are usually stored and available in the form of dormant stable cysts in the market, which can be conveniently hatched at needed amount one day before feeding **(Conceição *et al*., 2010)**. Among the Artemia strains used in aquaculture, *A. franciscana* produced in Vietnam has small size and high omega 3 content **(Clegg *et al*., 2000)**. AF *Artemia* has allowed the complete replacement of enriched rotifers which is bulky to produce and susceptible to bacterial contamination **(Battaglene *et al*., 2006)** or to shorten the rotifer-feeding period with 3 days for cobia, *Rachycentron canadum* larvae with insignificance impact on survival rate or on growth **(Nhu *et al*., 2009)**.

It is known that both rotifers as well as artemia are naturally lacking n-3 HUFAs, but linolenic acid rich and they must be enriched with n-3 HUFAs through algal diet to ensuring successful growth and enhanced larval metamorphosis **(Sargant *et al*., 1997; Holt, 2003; El-Sayed *et al*., 2021)**. The fatty acids ARA (arashidonic) and EPA (eicosapentanoic) play a very crucial role in the survival and growth of fish larvae **(El-Khodary *et al*., 2020)**. Moreover, their interaction induced eicosanoids formation that are essential for stress regulation, pigmentation, gonadal development, performance of spawning, and seeds quality **(Bruce *et al*., 1999)**.

Ornamental fishes are bought for their fascinating and attractive skin color and pattern. Moreover, the sexual selection depends to a large extent on color and diverse coloration patterns. These colorations are due to color pigments such as carotenoids **(Johnson *et al*.,1991)**. Pigmentation anemone fish usually starts during acclimatization process **(Elliott and Mariscal, 2001)**. These pigments cannot be stored beneath the skin permanently as fishes cannot form their own coloring pigments, the coloring agents which are created by some plants, specific algae, and probiotics, need to be included in their formulated diet **(Johnson *et al*.,1991)**.

Different enrichment strategies for live prey effect. with various nutritious algal strains, on larval production were studied. *Nannochloropsis oculata*, *Chlorella salina*, *Tetraselmis chuii* and *Rhodomonas salina* are highly recommended algal strains for life prey enrichment **(El-Sayed *et al*., 2014; El-Khateeb *et al*., 2021)**. These algae have historical reference in improving growth, survival, pigmentation as well as reducing oxidative stress and having high antimicrobial activity for fish larvae pathogens **(El-Sayed *et al*., 2014)**. It was also observed, in different fish species, that fishmeal and fish oil could be partially replaced (about 20 to 40%) by different types of microalgae such has *Isochrysis*, *Chlorella* meal, *Nannochloropsis* sp., *Tretraselmis* sp. and *Spirulina* without having a negative impact or even improving fish growth in various species **(Haas *et al*., 2016; Salin *et al*.,2018; Tibaldi *et al*., 2015)**. To the best of the authors' knowledge, no recent approaches or research work have been carried out concerning the impact of different enrichment strategies for live prey with various nutritious algal strains on clownfish larval production and coloration.

The current study endeavors to determine the requirements by the Clownfish *A. bicinctus* larvae**,** achieving a high survival rate and rapid development during larval cultivation in large scale under prospective conditions. The goals of this study also are 1) to explore the opportunity of complete substitution of rotifers by using AF *Artemia* and to evaluate its influence on growth and/or of *A. bicinctus* larvae stages.Therefore, the proposed approach of total alternating enriched- rotifers by AF *Artemia* nauplii may simplify the larval rearing protocol in case of unexpected drop of rotifer production or deficiency; and provide a more energetic food comparing with enriched rotifers. Additionally, 2) to assess four different algal enrichment for live fed (*Artemia*) including *R. salina* for the first time in larval production and find out the best growth, survivors, and onset coloration in larval clownfish.

MATERIALS AND METHODS

1. Brood stock maintenance

The brood stock was delivered to the Marine Aquarium of the National Institute of Oceanography and Fisheries, Alexandria branch five months pre-spawning season (in April). The brood stock was reared in a square fiber-glass tank of 750L volume with association laterally to their symbiotic anemone *Heteractis magnifica*, *H. crispa*, *H. aurora* and some rocks which are considered to be the substratum of spawning as described in **Abou Shabana and Helal (2006)**. The Brooders were bred in the same tanks where they were stocked in the first day of delivery that controlling the water quality as recommended condition for successful rearing that were described in **Abou Shabana and Helal (2006)** and **Helal and Abou Shabana (2008)** throughout the study period. Under the same conditions the fish spawned by a rate 2.4/month that usually decreased gradually with ending spawning season. About 350-400 embryos were grouped and were subjected to the parental care throughout the whole embryogenesis period. Hatching usually occurred, at 28 °C, 144 h after fertilization. An hour prior hatching, the rock or the substratum with the egg patch was stocked in 50 L larval raising tank provided by the similar physicochemical conditions of the parent tanks. An air stone was fixed close to the rock and the egg patch was placed in darkness for approximately 50 min. With ending this period hatching occurred. The hatchability ratio for the attained clutches was recorded 94±3%.

2. Experimental design

After the hatching, in experiment (1) the larvae were divided into two groups (stocked in 100 ± 5 larvae per tank, in triplicates each) in 50 L glass aquaria as follows in (Table 1), first experiment; group A: EROT fed enriched rotifers (*Brachionus plicatilis*) (10 ind/mL) with control *Nannochloropsis oculata* (as marine hatchery NIOF protocol) whereas group B: AF Art., fed AF 430 *Artemia* nauplii (Source: Inve Technologies, Belgium) (5 ind/ml) from day 1 to day 7 DAH. Starting from day 8 DAH, experiment (2) was applied containing Group C larvae (the rest larvae group that fed only on AF *Artemia* nauplii) that intern was divided into 4sub-groups C1,C2,C3 & C4 according to the enrichment algal strain for the second experiment as shown in table (1): Group C *Artemia* meta nauplii (EG brand) were enriched with *N. oculata* (for feeding Group C1) as control group, *Chlorella salina* (for feeding group C2) whereas, *Tetraselmis chuii* and *Rhodomonas salina* (for feeding Groups C3&C4), respectively. The water exchange was applied by gentle replacement ten times a day in the larval tanks by a dripping system. Moreover, the sides of the tanks were provided with black panels as covers to diminish light reflection, while the phytoplankton addition, as a green water technique, was used at an approximately maximum density (50.000 cells/mL) according to the algal strain; in order to condition the tank from day 7 to metamorphosis as described by **(Palmer *et al.*, 2007)**.

3. Larval sampling

At the end of each experiment sampling was performed at the morning prior to larval feeding for each treatment. Ten specimens were taken for morphometric analysis (total length (TL) and body weight). Larval length (a Stereomicroscope at a magnification of 0.67 to 3.5 × 10 while for the longer ones than 5 mm the ruler was used) and larval population (by counting larvae at end of each experiment). The larvae were irregularly sampled (n = 10) and measured at 1st DAH (first feeding), at 7th DAH (completion of 2 feeding strategies) and after 21 DAH (prior to weaning period) to assess growth and survivors in the different trials.

4. Live food preparation

N. oculata, *C. salina*, *T. chuii* and *R. salina* were maintained in carboys (20 L) using Conway medium (commercial ingredients) following standard protocols of **El-Sayed *et al*. (2014), El-Khateeb *et al*. (2020)** in enriched seawater at 25°C. *N.oculata* was added to the larvae tanks and used as food for the rotifers enrichment as recommended in the marine hatchery of NIOF and majority of marine hatchery protocols.

L-type rotifer (*Brachionus plicatilis*) was mass produced for the 1st experiment by culturing in 500L tanks by using baker yeast and *N. oculata* alga then enriched in separate tanks using *N. oculata* only for about 2-3 hrs before feeding to the larvae. Then filtering by a sea water to rinse enriched rotifers through cloth of 55µm mesh. Then part of it was used for feeding the larval rearing tanks, while the other part of rotifer was kept at 10–12 °C for maximum 8 h in 30L fiber glass tanks for extra feeding with addition of *N. oculata* at a density of 7×10^6cells/ml in order to provide ideal nutritional quality.

AF-brand *Artemia* cysts (Source: INVE Aquaculture SA, Belgium) were sterilized and raised at 28–30 °C in 30 g/L seawater under continuous illumination condition and vigorous aeration **(Lavens and Sorgeloos, 1996)**.

5. *Artemia* Enrichment

EG-brand *Artemia* cysts (Source: Great Salt Lake USA, INVE Aquaculture SA, Belgium) were chosen for the second experiment that were sterilized and hatched as described by **Lavens and Sorgeloos (1996)**. Then the metanauplii were enriched with different algal enrichments (*N. oculata*, *C. salina*, *T. chuii* and *R. salina*) which maintained at their appropriate culture conditions as described above in continuous cultures which harvested at the end of the exponential phase. The enrichment protocol and the green water technique were applied following **El-Sayed *et al*. (2014)** and **El-Khodary *et al*. (2020)**.

6. Fatty acids determination

After enrichments, *Artemia* metanauplii were rinsed with filtered seawater and were concentrated in small containers then put in storage at-80°C till further analysis. Total lipids were separated from the enriched Artemia by applying **Bligh and Dyer (1959)** method and the analysis of fatty acid was done using gas liquid chromatography (GC-QqQ/MS triple Quade) analysis system (Agilent Technologies Inc., USA) according

to the standard protocol defined in **Doan *et al.* (2011)** and were estimated in mg/L dry weight then calculated individually as a fraction of the total fatty acids.

7. Evaluation of prey size with larval mouth opening

The sizes of rotifer loricae were detected as described by **Fu *et al.* (1991)** as well as AF *Artemia* nauplii at hatch, in addition to fish larval mouth opening were evaluated under a microscope to assess and check for live feed intake by *A. bicinctus* larvae 1st feeding. Feeding capture rate was determined by investigating 20 Clownfish newly hatched larvae collected randamly at the 1st feeding day as test before trying at larval tank.

8. Statistical analysis

For the first experiment, deferences between growth (length &weight) and survival of *A. bicinctus* means of the two experimental groups (a and b) were compared by student's T-test. For the second experiment, feeding incidence, growth performance, survival of *A. bicinctus* among the treatments, also fatty acid content of *Artemia* enriched with different treatments were verified for significance differences ($P<0.05$) using One-way ANOVA, Differences were considered significant at 0.05 probability level. Comparing of means done using Duncan's multiple range test (DMRT) **(Zar, 1999)**. All statistical assessments were achieved with SPSS 20.0 software.

RESULTS

1. Size suitability and digestibility of AF *Artemia*, growth and survival of *A. bicinctus* larvae

In the first trial, the NEH *A. bicinctus* length varied from 4.49 to 5.27 mm with a mean of 5.0 ± 0.16 mm, having a mouth opening sized 523.0 ± 4.7 μm. The newly hatched AF *Artemia* ranged between 387.5-411.6 μm in length with an average of 399.55 ± 0.2 μm, while the rotifer loricae ranged between 100.0 ± 3.0 μm and 150.0 ± 4.0 μm. However, AF *Artemia* nauplii were surpassed rotifers in size, they were more fitting and early detectable in the larval digestive tract approving their ingestion. For the growth of *A. bicinctus*, at the end of 7 DAH results showed that no significant ($P<0.05$) was detected amongst group A and group B for larval growth and survival in Table (2). Comparing to larval total length and weight by T test displayed that no significant growth differences were observed for larvae fed with AF *Artemia* or enriched rotifer *B. plicatilis*after 7 days of experiment. Also, survival of *A. bicinctus* displayed no significant difference between groups A and B when compared by T test as shown in Table (2).

Table 1. Feeding Regimes schedules for the two experiments

Groups	(1-7) DAH	8 DAH to metamorphosis
Exp.1		
Group A	Fed with EROT (10ind/ml)	-
Group B	Fed with AFArt (5ind/ml)	-
Exp.2 Group C	Ended with feeding AF Art (3-5ind/ml).	Fish fed with *Artemia* nauplii at Instar 2 enriched with *N. oculata*.
C1	-	Fish fed with *Artemia* nauplii Instar 2 enriched with *C. salina.*
C2	-	Fish fed with *Artemia* nauplii Instar 2enriched with *T. chuii*
C3	-	Fish fed with Artemia nauplii Instar 2enriched with *R. salina*
C4		

Group (A) EROT: fish fed with enriched rotifer
Group (B) AF Art: fish fed with newly hatched AF *Artemia*
Group (C) Art: fish fed with enriched *Artemia* metanuplii EG

Table 2. Growth and survival of newly hatched *A. bicinctus* fed with AF *Artemia* (AF Art) and enriched rotifer *B. plicatilis* (EROT)

Treatment	Initial length 1st DAH	Final length at 7 DAH (mm)	Initial weight 1st day feeding (mg)	Final weight At 7 DAH (mg)	Survival (%)
AF Art	5.4±0.14	8.3±0.6	40 ±0.25	60.5±0.45	71.7 ± 11.1
EROT	5.4±0.16	8.1±0.6	40 ±0.23	60.0 ±0.46	68.9 ± 12.3

Values presented as mean±S.E , data compared for the two experimental groups by T-test (P<0.05).

2. Growth, survival and pigmentation of *A. bicinctus* larvae fed on algal enriched EG *Artemia*

For the second experiment, the enrichment by four different microalgae from 8 DAH till 21 DAH highlighted that Larvae of initial weight (60mg) fed with *R. salina* enriched *Artemia* (C4) reached the highest weight of 111.06 ± 0.2 mg, than *N. oculata* (C1) with a weight of 106.9 ± 1 mg (Table 3). The same trend was observed for the total final length among treatments.

The Survival percentage of *A. bicinctus* revealed that *R. salina* enriched with *Artemia* (C4) indicated the greatest survival of 80 %, after that *N. oculata* (C1) with 75% then *T. chuii* (C3) 70 % and lowest survival (68 %) by *C. salina* (C2) as shown in (Table 3).

The larvae growth after 21 DAH for different treated groups is demonstrated in Table (3). Larvae fed with *R. salina* enriched *Artemia* (C4) exhibited the maximum weight gaining (51.06 ± 1 mg) followed by larvae fed with *N. oculata* enriched *Artemia* (C1) (46.92 ± 1.0mg) whereas *T. chuii* and *C. salina* enriched *Artemia* recorded the lowest values (38.74 ± 0.4 & 33.21 ± 0.04mg, respectively). These results matched with the same trends of data obtained for length gain and SGR % as shown in Table (4). Otherwise, the days number elapsed for the onset pigmentation (*i.e.*, the starting time in which the first pigmentation characteristic of the species appeared) was reported for larvae fed with various enriched diets. It was noticed that in *R. salina* enrichment, first *A. bicinctus* larvae pigmentation appeared after 3days of experiment (11DAH), while it took 3-4 days (11-12 DAH) in *T. chuii* treated larvae, whereas it extended to 4-5 days (12-13 DAH) & to 5-6 days (13-14 DAH) in fish fed *N. salina* and *C. salina* enriched *Artemia* respectively (Table 4). Also Table (4) displays the number of days taken to show early signs of metamorphosis according to different enriched diets. *R. salina* treatment took only 9 days (17 DAH), subsequently 10 days (18DAH) in *N. salina* and got a longer time in larvae fed with *T. chuii* and *C. salina* enriched *Artemia*.

Table 3. Growth and survival of *A. bicinctus* larvae at different microalgal species treatments at optimum water rearing conditions form 8- 21 days after hatch (DAH)

Diet	Temp.	Salinity	pH	No. of larvae	Initial weight (mg)	Final weight mg	Initial Length (mm)	Final Length mm	Survival rate (%)
N.oculata	27±1	36±2	7.76	100	60±0.45	106.92±0.2^{b}	8.4±0.36	13.8±0.43^{b}	75.0±2.9^{b}
C.salina	27±1	36±2	7.76	100	60±0.43	93.12±1.1^{c}	8.4±0.35	12.3±0.54^{d}	68.0±2.7^{d}
T. chuii	27±1	36±2	7.85	100	60±0.48	98.74±1.8^{b}	8.4±0.33	12.9±0.45^{c}	70.0±4.3^{c}
R.salina	27±1	36±2	7.87	100	60±0.44	111.06±0.2^{a}	8.4±0.37	14.1±0.56^{a}	80.0±2.4^{a}

Values (mean ± SE), not the same super script letterswithin the same column are significantly different (P<0.05)

Table 4. Growth performance, onset pigmentation and initial metamorphosis stages (days from experiment start) of *Abicinctus* fed EG *Artemia* enriched with *N. oculata, T. chuii, C. salina* and *R. salina* for the period from (8- 21) DAH.

Enrichment media	Weight gain (mg)	Length gain (mm)	SGR%	Pigmentation (days)	Metamorphosis (days)
N. oculata	46.92	5.4	4.13	4-5	10
C. salina	33.12	3.9	3.50	5-6	12
T. chuii	38.74	4.5	3.11	3-4	11
R.salina	51.06	5.7	4.40	3	9

SGR: specific growth rate

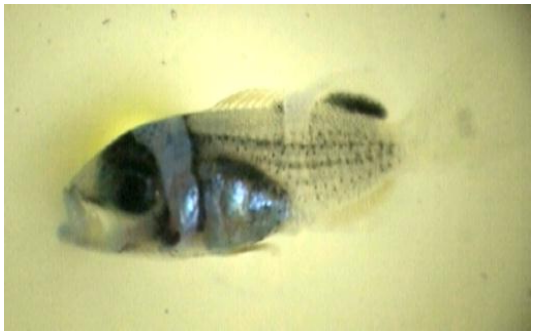
After hatching, *A. bicinctus* larvae measured about 5.0 ±0.16mm in total length with a minor yolk sac, translucent body displaying one single fin fold; also the mouth was

opened and dispersed pigmentations were detected on the body with increasing strength on the line along the vertebrae (lateral line) and close to the gut area (Figure 1a). At 8 DAH pigmentation was significantly observed on head and gut areas, fin fold disappeared progressively and distinct anal, dorsal, and pelvic fin rays were observed (Figure 1b). Larvae pigmentation by the end of second trial (21 DAH) was greatly affected by the type of algal enrichment. *A. bicinctus* larvae fed with *N. oculata*enriched *Artemia* (C1 group) body were faint yellow in color with faint black on the fins, white bands are clear and sharp (Figure 1c). *A. bicinctus* larvae fed on *C. salina* enriched *Artemia* (C2 group) were transparent with faint white bands and caudal fin was transparent with no black color spot (Figure1d). *A. bicinctus* larvae fed with *T. chuii* enriched *Artemia* (C3 group) were very faint in color but white band were clear, caudal fin is transparent with no black color (figure1e). pigmentation of *A. bicinctus* fed with *R. salina* enriched *Artemia* (C4 group) displaying the best coloration comparing to the other three treatments; two white bands broadened and appear clear (one on the opercula region near the eye and other white band in central of the body just next to the dorsal fin, also black color appeared on dorsal and caudal fins and on head above the eye, larvae were predominantly yellow-orange in color (Fig.1f).

(a) Newly hatched larva day zero with (Magnification X 3.5)

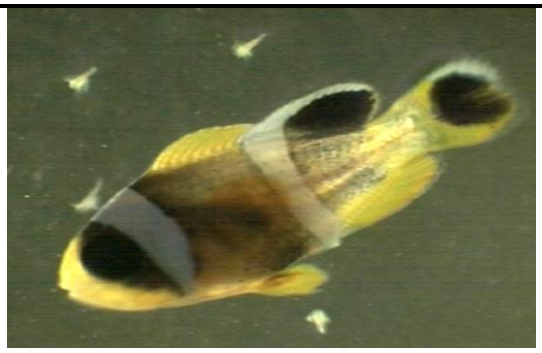
(b) Larva of *A. bicinctus* 8 days after hatching in fed AF *Artemia* nauplii (magnification x 2.5)

(c) Larva of *A.bicinctus* 21 days after hatching *N.oculata* (magnification X 1.5)



(d) Larva of *A.bicinctus* 21 days after hatching fed with *C. salina*-enriched artemia (magnification X 1.5) showing onset pigmentation

(e)Larva of *A.bicinctus* 21 days after hatching fed with *T. chuii* –enriched artemia (magnification X1.5)



(f) Larva of *A.bicinctus*21 days after hatching fed with *R. salina* enriched artemia (magnification X1.5)

3. Fatty acid profile of the enriched Artemia (EG)

The Fatty acid composition of EG *Artemia* metanauplii enriched with *N. oculate* (NEA), *T. chuii* (TEA), *C. salina* (CEA) and *R. salina* (REA) is given in Table (5). The enrichment effects on fatty acids contents revealed highly significant levels ($p<0.01$) among the different treatments. As depicted in the Table the results showed the highest contents of total unsaturated fatty acids, HUFA, PUFA and n-3 levels as well as EPA/AA ratios in REA followed by TEA whereas CEA registered zero ratio due to the absence of EPA in *C. salina* profile. The data also declared that DHA/EPA ratio in particular was high in REA and NEA treatments (0.2 ± 0.1 & 0.14 ± 0.02, respectively). Simultaneously TEA and CEA recorded no value due to lacking of DHA fatty acid in *T. chuii* and *C. salina* and in *Artemia* EG itself.\

Table (5). Grouping for total fatty acid fractions of *Artemia franciscana* (EG)enriched with various diets (% of total fatty acid).

Enrichments	Σ Unsaturated	HUFA	PUFA	n-3	DHA/EPA	EPA/AA
N. oculata	45.22 ± 1.2^{b}	20.48 ± 0.3^{d}	25.54 ± 0.3^{c}	17.3 ± 4.30^{c}	0.14 ± 0.02^{b}	2.72 ± 0.28^{c}
C.salina	46.74 ± 1.1^{b}	22.03 ± 0.6^{c}	24.71 ± 0.2^{d}	8.9 ± 3.60^{d}	---	-
T.chuii	63.20 ± 0.8^{a}	28.0 ± 0.4^{a}	35.2 ± 0.4^{b}	34.2 ± 0.03^{b}	--	8.23 ± 0.20^{b}
R.salina	62.90 ± 1.8^{a}	26.8 ± 0.2^{b}	36.1 ± 0.8^{a}	34.5 ± 0.20^{a}	0.20 ± 0.10^{a}	8.9 ± 0.40^{a}

values (mean ± SE) with different superscript letter swithina column are significantly different **($P<0.01$).** The individual fatty acid is expressed as the percentage of total identifiable fatty acids**.**

Σ Unsaturated = total unsaturated fatty acids; AA= Arachidonic acid 20:4 (n−6); EPA= Eicosapentaenoic acids (C20:5); DHA= Docosahexaenoic acid (C22:6); HUFA= Highly Unsaturated Fatty Acids (monounsaturated fatty acids in particular); PUFA=Poly Unsaturated fatty acids;n3=Omega-3 fatty acids.

DISCUSSION

The current study attempts to set up the best necessities by the clown fish *Amphiprion bicinctus* attaining a better survival and developmental signs during cultivation in the captive laboratory conditions**.**

The present study demonstrated that *A. bicinctus* larvae at hatch having initial total length of 5.0 ± 0.16 mm with a mouth opening of 523.0 ± 4.7 μm, were eligible to ingest the newly hatched (NEH) AF *Artemia* of 399.55 ± 0.2 μm in length, proposing that AF *Artemia* is suitable to fed NEH of *A. bicinctus* replacing enriched rotifer (EROT) with no significant impact on growth or survival till 7 DAH. The mouth of Clownfish was well developed in hatched larvae or within few a hours after hatching, according to **Madhu *et al*. (2013)**. Clownfish *Amphiprion percula* NEH larvae had a length ranging from 3.2 to 3.6 mm with mouth an opening ranging from 300 to 400 μm and were able to start the first feeding. Moreover, redhead dottyback**,** *Pseudochromis dilectus* started first exogenous food at (10 -12) hours after hatch **(Madhu *et al*., 2016)**. As well as for *A.bicinctus*, the NEH larvae had well developed tail fins and started to manipulate food from water just after few hours after hatching as the mouth opened within six hrs. after hatching **(Abou Shabana and Helal, 2006)**. For black *Amphiprion ocellaris,* yolk sac exhaustion took place around (5 – 7 hrs.) after-hatch and NEH larvae displayed well developed mouth **(Raheem *et al*., 2021)**. In settlement with our results, **Nhu *et al*. (2009)** recommended that cobia larvae can intake and assimilate AF *Artemia* since first feeding. Moreover, changing EROT as starter meal for cobia larvae by using AF *Artemia* has very little influence on increase through eight DAH and seems to reflect no significance impact on seed quality or on growth as well as survival by 18 DAH. Similarly, the mouth size of the seabass, *Dicentrarchus labrax* larvae is wide enough to match brine shrimp nauplii at first feeding and these larvae do not need the small rotifers as first prey**,** in contrasting sea bream–hatcheries cannot dispense producing rotifer as initial feed **(FAO, 1999)**. Additionally, AF *Artemia* can be introduced to sole, *Solea senegalenesis* larvae directly pre-hatching and it can induce a good feeding response due to its bright color **(Sá, 2016)**. Although, AF *Artemia* has high cost compared to the EG strain, it possesses high HUFA content at hatching without needing further enrichment **(Sá, 2016)**. The effective use of AF *Artemia* instead of rotifer at early life of *A.bicinctus* launches an opportunity to facilitate its larval rearing handling and reduce the live food scaling up expenses**.**

In the present work, *Artemia* enrichment by four different microalgae indicated the *R. salina* enrichment comparing to the other three microalgae, significantly increased the length, weight and survival of *A.bicinctus* larvae by the 21 DAH. Moreover, *Rhodomonas salina* accelerated pigmentation and metamorphosis. To the best of the authors knowledge, this is the first time to use *R. salina* enriched *Artemia* to fed ornamental fish.

Microalgae biomass considered as valuable feed substitute as it contains low to medium protein levels (about 43 -60% of dry matter), depending on species, culture and

harvesting conditions **(Becker, 2007; Biller and Ross, 2011)**. In addition, microalgae are considered a main source of protein and lipids and are rich in natural carotenoids that act as a natural coloring agent **(Gouveia *et al*., 2006).**

Using of *Rhodomonas* sp. for live fed enrichment in combination with other microalgae achieved best larval growth and survival **(Seixas *et al*., 2010)**, *Rhodomonas* sp was used for DHA enrichment **(Dhert *et al*., 2001)**. Previous work on molluscs filtration have proven that *R. salina* constitutes highlighted food quality for *Crassostrea gigas* spat **(Brown *et al*., 1998)**, and increases culture productivity and induces the metamorphosis rate of *Pecten maximus* when used as an enrichment against other "standard" algae used in hatcheries **(Tremblay *et al*., 2007)**.

In the present work, enrichment by microalgae *N. oculata* was the second significant effective algal enrichment after *R. salina, N. oculate* enrichment increased the length, weight and survival, accelerated pigmentation and metamorphosis of *A.bicinctus* larvae but with less significance compared to *R. salina*. In marine ornamental fish larviculture, live microalgae *N. oculata* is frequent options of microalgae for producing greenwater circumstance **(Olivotto *et al*., 2006; Baensch and Tamaru, 2009; Leu *et al*., 2009; Olivotto *et al*., 2010; Moorhead and Zeng, 2011)**.

For Clownfish, *Amphiprion nigripes* larvae achieved high growth and the highest survival rate observed when fed on algae EROT more than those supplemented with poly unsaturated fatty acid **(Ajith Kumar *et al*., 2012)**. Also, algae EROT attained larvae metamorphosed during range 15-17 days with taking parent coloration **(Ajith Kumar *et al*., 2012)**. On the other hand, *Amphiprion ocellaris* shows positive results with the maximum survival, growth and the metamorphosis when fed EROT by n-3 PUFAs emulsion incorporated with ingredients (vitamin C, carotenoid, and antioxidant) than those fed on algae EROT. In rotifer, *B. plicatilis* algae have been used to match the omega 3 fatty acids like (EPA) and (DHA) by long term enrichment and feed the fish larvae on the next morning **(Agh and Sorgeloos, 2005)**. Better immunity and higher strength of the fish larvae have been significantly increased by feeding of algae EROT **(Ajith Kumar *et al*., 2012)**. It was stated that larval mortality of Clownfish is mainly attributed to the non-availability of suitable size feed and to their nutritional deficiency via essential fatty acids especially on the 2nd and 7th DAH **(Ignatius *et al*., 2001)**.

The main problem in rearing ornamental fish is the loss of color due to carotenoid deficiency in diets; therefore, the use of microalgae in fish diets has various benefits that make them an interesting ingredient to be included in fish nutrition especially in the ornamental market where the color is one of the limiting factors of the trade **(Morais, 2019)**. Nowadays microalgae are one of the most used sources of natural carotenoids. The harvesting time of microalgae can determine the desired color pigment that say if the desired color is green the harvest should be done very early whereas for orange-red, carotenogenesis phase must occure over time and harvesting must be performed for biomass on a late exponential **(Gouveia *et al*., 2006)**. According to **Hekimoğlu *et al*.**

(2017), the addition of microalgae species such as *N. oculata*, *P. cruentum* and *Spirulina* inclosing natural pigments agents to the feeds which maximize the total amount of carotenoids in Clownfish skin. Therefore, nutrition is a valuable tool to improve the quality of the reared ornamental fish **(Morais, 2019)**.

CONCLUSION

In conclusion, it is feasible for *A.bicinctus* larvae ability to intake , assimilate and excrete AF Artemia starting from first feeding. Replacing EROT with AF *Artemia* as first feed has no effect on growth and survival by 7 DAH or a negative effect on larval quality, by 21DAH. The effective use of AF *Artemia* simplifies the larval rearing complexity for *A.bicinctus* fish, through lowering of labor costs for live food scaling up. Moreover, our study recommends the microalgae, *R. salina* for Artemia enrichment (EG)for the ornamental fish larvae, *R. salina* enrichment achieved the highest survival, growth, pigmentation and metamorphosis by 21 DAH. It is crucial to include microalgae in diets of ornamental fish to improve fish coloration, which is the most desired trait in the market.

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