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Breeding and Hybridization of Clownfish *Amphiprion ephippium* × *Amphiprion melanopus* in Captivity

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ABSTRACT

Development of broodstock, spawning, and early rearing of the hybrid clownfish, *Amphiprion ephippium* (♂) × *Amphiprion melanopus* (♀), was studied under captive conditions. The fishes were successfully paired after being together for over a year. Spawning occurred between 0700 to 1000 hours every 3 weeks. More than 200 eggs were spawned each time, with an egg size of 1.5 ± 0.5mm. Fertilized eggs turned from bright orange to black to silvery before hatching after being incubated for 7–9 days. Out of 20 batches of eggs spawned, 5 batches were successfully hatched, with only 2 batches surviving to adulthood. The average survival rate for all the batches hatched was 21.16%. Newborn larvae measured about 3–4 mm long, with transparent fins that fused, forming a single fin fold. Larvae underwent metamorphosis on day 10 post hatched, where the fins started to separate, form, and develop body colorations. Two clear and thick bands were observed on the body (head and middle) as early as 14-day post hatched to 90-day post-hatched, where the banding reached its peak. The middle band then began fading as the juveniles grew. By 130-day post-hatched, the juveniles became adults with unique coloration featuring a headband and a black blotch, reaching the maximum size of 34 mm. The hybrid clownfish underwent metamorphosis earlier and reached marketable size much sooner compared to its parent species, making it a suitable candidate for ornamental fish culture. It is the first documentation on the production of hybrid clownfish *A. ephippium* and *A. melanopus* both in Malaysia and worldwide.

Keywords: Captive breeding, hybrid clownfish, juvenile rearing, Malaysia, marine ornamental

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INTRODUCTION

Aquaculture has been identified as a priority sector in the development of Malaysia's economy (Jumatli & Ismail, 2021). Its ornamental fish industry also played a significant role in the national economy, and this industry is now enjoying rapid growth (Othman et al., 2017). One of the most demanding marine fish in the aquarium trade is the clownfish or anemonefish (*Amphiprion* spp.). However, most of these species are wild-caught, increasing the chance or potential for the fish to be overharvested (Dhaneesh et al., 2012; Domínguez & Botella, 2014). One way to reduce the pressure on the ecosystem and meet the increasing demands for marine ornamentals is to improve the efficiency of the capture and the culture system for desirable marine species (Domínguez & Botella, 2014; Noh et al., 2013). Rearing fish in closed systems reduces pressure on wild populations and is likely to produce harder species that are far better in captivity and survive longer (Wittenrich, 2007). Today, as many as 18 clownfish from *Amphiprion* have been reared in captivity. However, their larvae rearing is still time-consuming and expensive (Arvedlund et al., 2000). Hence, knowledge of the life history of the species under study is crucial to the success of captive breeding (Olivotto et al., 2011).

Hybridization or crossbreeding has been a powerful tool to improve production and breeding qualities in fish (Chapman & ZoBell, 2010). Hybridization in ornamental fish was mostly performed on freshwater fish, although recently, there has been an attempt to crossbreed different species of clownfish (*Amphiprion percula* × *Amphiprion ocellaris*) and has been proven successful (Balamurugan & Kathiresan, 2018). Hence, there is a potential to develop hybrids with other clownfish species. Red saddleback anemonefish (*A. ephippium*) and fire clownfish (*A. melanopus*) are potential candidates. These two species can be found in Malaysian waters (Department of Fisheries Malaysia, 2009), while *A. ephippium* is considered a native species in Malaysia (Jenkins, Carpenter, et al., 2017). Both species were collected in the aquarium trade (Jenkins, Allen, et al., 2017; Jenkins, Carpenter, et al., 2017), causing the species to be overfished in localized areas and potentially endanger the local population (Domínguez & Botella, 2014).

Nowadays, clownfish breeding and conservation are very important to cater to the increasing demand in the ornamental trade. However, to our knowledge, the culture of hybrid clownfish has not yet been reported in Malaysia, despite Malaysia being one of the main exporters of marine ornamental fish (Rhyne et al., 2017). Furthermore, there is no report on the embryo development, larval-rearing methods, and production of any clownfish juveniles in Malaysia. Thus, this study will likely be the first documented nursery culture of hybrid clownfish of *A. ephippium* (♂) and *A. melanopus* (♀). This study was designed to determine the larval development and survival rate of hybrid clownfish of *A. ephippium* (♂) and *A. melanopus* (♀).

MATERIALS AND METHODS

Attaining Fish Samples and Holding Tank

Live juveniles of *A. ephippium* and *A. melanopus* bought from a local aquarium shop were used in this study. Experiments were conducted at an indoor and outdoor nursery of Fisheries Research Institute (FRI) Batu Maung in Pulau Pinang, Malaysia, from August 2017 to March 2020. The tanks were supplied with filtered seawater drawn from the treated reservoir tank. Continuous aeration was supplied, and 10–20% water exchange was done on alternate days. Water quality parameters such as dissolved oxygen (DO), pH, ammonia (NH_3), nitrate (NO_3^-), phosphate (PO_4^{3-}), and salinity were maintained and measured every 2 weeks (Table 1). For indoor tanks, the water temperature was kept at 28 °C and controlled by a 200 W submersible thermostat heater. A photoperiod of 10 h light and 14 h dark (lights on at 7 a.m. and lights off at 5 p.m.) were maintained throughout the study. For outdoor tanks, room temperature and natural photoperiod were provided. Both juveniles were reared and kept together in the same nursery tank (92 cm L × 46 cm W × 46 cm H) until pairs were formed.

Table 1
Water parameters maintained in the tanks

Parameter	Range
Dissolved oxygen (mg L^{-1})	5.78–6.12
pH	8.0
Ammonia (mg L^{-1})	0–0.5
Nitrate (mg L^{-1})	0
Phosphate (mg L^{-1})	0–0.5
Salinity (ppt)	31–35
Temperature (°C)	27–29

Experimental Design – Breeding Tank

Fish-forming pairs were transferred to a breeding glass tank (61 cm L × 46 cm W × 46 cm H). Substrates of dead coral chips and live rocks were provided at the bottom of the tank to mimic the reef environment. Flat rocks were used as a substrate on which the fish could attach their eggs to the surface. The pair was fed a combination of live adult *Artemia*, live mysids, and pellet marine food twice a day. Light emitting diode (LED) light was fixed on top of the tank to provide a light intensity of 3,000–4,000 lux for 10 h daily. After the pair deposited their eggs on the rock substrate, the rock with egg clutches will be removed into the rearing tanks one day before hatching.

Experimental Design – Rearing Tank

For the first 17 hatching events, the hatching tank used was an indoor squared glass tank (25 cm L × 25 cm W × 25 cm H) before changing to an outdoor cylindrical fiber tank (80 cm in diameter). The rock with the egg clutch was propped diagonally against the tank wall, with an air stone placed directly under the rock to aerate and agitate the eggs to aid in hatching. No other substrate was added inside the tank. All live newborns that hatched were then collected, counted, and transferred to a rearing tank (batches 1 to 17 in an indoor rectangular glass of 40 cm × 25 cm × 25 cm in size: batches 18–20 in an outdoor rectangular fiber tank of 2 m L × 0.5 m W × 0.5 m H in size). The sides of the indoor tank are covered with black panels to reduce light reflection. All tanks were aerated gently. Feeding began on the first day of hatching, starting with green algae of *Nannochloropsis* sp. The juveniles were fed different types of diets, as in Table 2.

Table 2
Type of diets for hybrid clownfish according to days after hatching

Day	Food
1	Green algae (*Nannochloropsis* sp.)
2–10	Rotifer
11–30	Enriched rotifer + newly hatched *Artemia*
31–60	Enriched *Artemia* + small-sized pellet food
60 onwards	Marine pellet food

During the study, conditions in all tanks were kept the same. No substrate was used in the rearing tanks. The bottom of the tanks was cleaned, dead juveniles were removed, and 20-25% water exchange was carried out daily. A minimum of ten juveniles was randomly selected, and their total length and wet weight were measured. Measurements were made every 30 days for 6 months. Days of metamorphosis were noted. A gross estimate of the survival rate in all batches was made by counting the fish larvae in each batch at the time of hatching and the end of the experiment using the following formula:

Survival rate (%) = (Number of larvae surviving/Number of larvae hatched) × 100%

RESULTS AND DISCUSSION

Spawning and Broodstock Development

After a year in captivity, the two fish began to form a pair bond. *A. melanopus* developed into a functional female, while *A. ephippium* became the functional male. Within a few months, the pair began to spawn. A day or two before spawning, the male *A. ephippium* cleaned the nest area. Spawning occurred approximately every two weeks and was observed to take place mostly between 0700 to 1000 hours. More than 200 eggs were spawned at a time, with an initial size of about 1.5 ± 0.5 mm in length, and were usually deposited on the flat rock (Figure 1). The male *A. ephippium* played an important role in the incubating stage, where it was observed to be aerating and guarding the eggs until they hatched. The eggs were initially bright orange before eventually turning transparent, where the eyes of the larvae can be seen developing within the egg case. The eggs normally hatched between days 7 to 9.

The pair spawned every month and produced an average of 2.2 egg clutches per month. A total of twenty batches of eggs from the same broodstock were obtained within 9 months (December 2018 to August 2019). However, only five batches of eggs managed to hatch successfully, and three

Figure 1. Amphiprion melanopus laying eggs on a flat rock

Table 3
Survival rates for batches that are successfully hatched

Batch	Date of laying eggs	Date of hatching	Incubation (days)	No. of hatchlings	No. of hatchlings survived	Survival rate (%)
3	18/01/19	27/01/19	9	1	0	0
9	10/04/19	19/04/19	9	1	0	0
18	12/07/19	19/07/19	7	52	44	84.62
19	22/07/19	29/07/19	7	61	3	4.92
20	16/08/19	22/08/19	7	7	0	0

had fewer hatchlings (less than 10). Only two batches survived and reached adult size (Table 3). The average survival rate for all the batches that hatched was 21.16%, with only batch 18 reaching 84.62%, while the rest (batches 3, 9, 20, and 19) were 0% and 4%, respectively.

Hybrid Larval Development

It is well established that the life cycle of most reef fishes can be divided into three distinct biological/ecological phases: larvae, juvenile, and adult (Olivotto et al., 2011). In this study, the larvae phase started after 7 days of incubation, which is the same case for most species of clownfish, such as *Amphiprion ocellaris* (7 days) (Balamurugan & Kathiresan, 2018), *Amphiprion melanopus* (7–8 days) (Uthayasiva et al., 2014), and *Amphiprion akallopisos* (7–9 days) (Dhaneesh et al., 2012). However, Rohini Krishna et al. (2018) reported that the hatching of *A. ephippium*'s eggs occurred as early as the sixth day of incubation.

The first batch of newborn hybrid clownfish was hatched in early January 2019. The larval was measured about 3–4 mm long. Its large dark eyes, open mouth, and internal organs can be seen through its transparent body. The fins were transparent and fused, forming a single fin fold. High initial mortality was commonly observed before the metamorphosis phase. Metamorphosis of the larvae started at day 10 post hatched when the fins started to separate and form as well as develop colors (Figure 2).

The newly hatched larvae were fed with algae as first feed and later with rotifers and finally *Artemia nauplii*. Rohini Krishna et al. (2018) reported that larvae of *A. ephippium* started feeding actively from the first day onwards. The given diets included rotifers, *Artemia*, and pellet feed. Algal-enriched

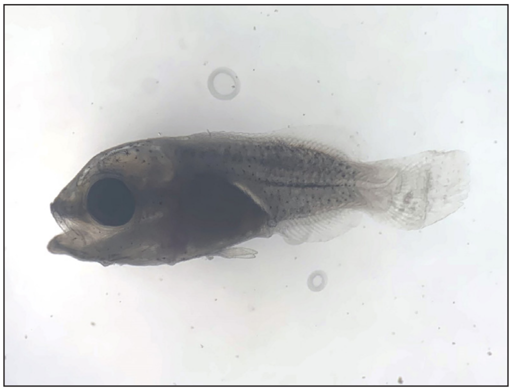

Figure 2. Microscopic observation (× 4.5) of ten-day-old larvae of hybrid *A. ephippium* and *A. melanopus*

rotifers and *Artemia* nutrition represent the underlying foundation for successful larval rearing. The difference in food quality may also play a role (Arvedlund et al., 2000). High survival rates were observed in larvae fed exclusively on enriched rotifers and *Artemia* (Olivotto et al., 2011), the same proven in this study for the hybrid clownfish larvae. After the metamorphosis stage, juvenile rearing was generally non-problematic if live feeds were enriched properly and good water quality was maintained.

The transformation of clownfish juveniles can be quite abrupt, with most clownfish species larvae starting to metamorphose 14 days after hatching (Wilkerson, 2001). However, the hybrid clownfish larvae started metamorphosis as soon as day 10 after hatching. The juveniles were observed to have developed well-defined fins (dorsal, pelvic, and caudal fins) their bodies turned dark. These results were also seen in reports by Rohini Krishna et al. (2018) on one of its parent species, *A. ephippium* (10 days after hatching). Its other parent species, *A. melanopus*, also reported undergoing a metamorphosis as early as day 8 after hatching (Arvedlund et al., 2000; Green & McCormick, 2001), which might contribute to the hybrid larvae starting to change faster than other species of clownfish, such as *Premnas biaculeatus* (11–12 days) (Madhu et al., 2006), *Amphiprion chrysogaster* (12–15 days) (Gopakumar et al., 2001), *Amphiprion sebae* (12–15 days) (Ignatius et al., 2001), *Amphiprion percula* (13-15 days) (Dhaneesh et al., 2009), and *Amphiprion akallopisos* (15–16 days) (Dhaneesh et al., 2012).

On day 15, post-hatched, the headband was visible with the fins, and body coloration turned red, resembling *Amphiprion frenatus*. At day 30 post-hatching, the headband was very clear, and the middle band could be seen from the dorsal part of the body, which was starting to take shape. The middle band has formed and reached half of the middle body at day 60 post-hatching. Two clear and thick bands were observed on the body (head and middle) at 90 days post-hatched, where the banding peaked. After that, the middle band began fading as the larvae grew, leaving only a white spot at the middle dorsal body by 120 days post-hatching. After that, the body color changed to dark red, resembling the color of an adult *A. melanopus*. The middle band completely disappeared by 130 days post-hatched, and a black blotch was seen at the sides when transitioning to adults with a maximum size of 34 mm. The adult hybrid clownfish has a clear headband resembling *A. melanopus* and a black blotch on the side and fin colors resembling *A. ephippium*, creating a new body pattern and coloration different from its parents (Figure 3).

The body transformation of the hybrid clownfish larvae closely followed the transformation of *A. ephippium* as reported by Rohini Krishna et al. (2018) in the early stages, whereby day 14 after hatching, larvae of hybrid clownfish and clownfish *A. ephippium* had the adult body coloration and clear headbands. Both also showed a clear middle band forming at day 30 post-hatched.

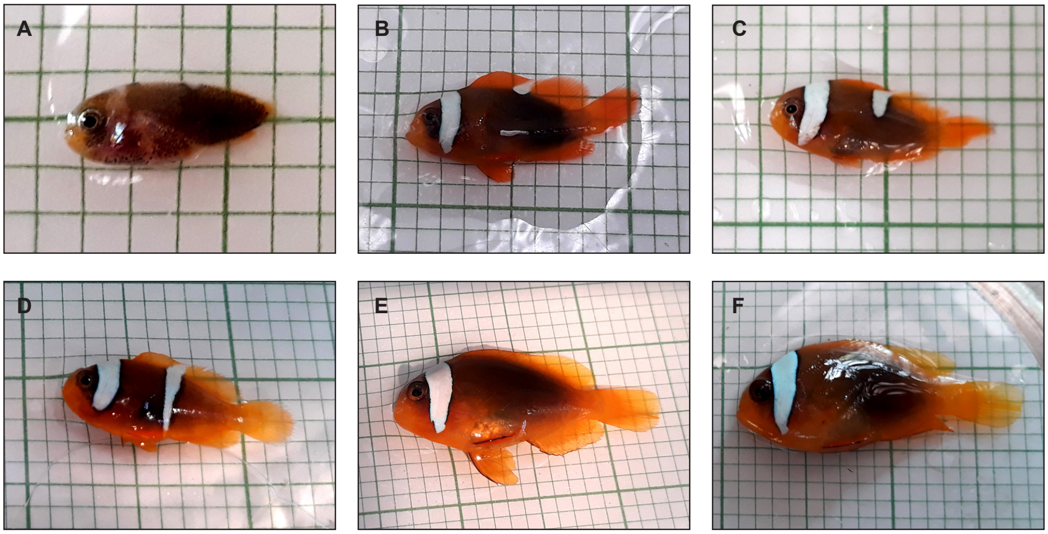

Figure 3. Larval development of hybrid *A. ephippium*♂ and *A. melanopus*♀. A: day 15 post-hatch; B: day 30 post-hatch; C: day 60 post-hatch; D: day 90 post-hatch; E: day 120 post-hatch; F: day 150 post-hatch. Each grid box represents 2mm in length

However, the peak banding for *A. ephippium* was reported at day 45 post hatched, much earlier than the peak banding of the hybrid clownfish 90 days post-hatch. Moreover, the middle band completely disappeared in *A. ephippium*, resembling the adult *A. frenatus* by 120 days after hatching. In contrast, remnants of the middle band were still present in the hybrid clownfish, and its body coloration turned darker, resembling *A. melanopus* 120 days post-hatch.

The headband in *A. ephippium* continued to fade as the larvae grew until it disappeared 310 days after hatched. Compared to its parent species, the hybrid clownfish larvae did not lose the headband as it grew and developed the black blotch on the side instead (a feature that distinguished the parent species *A. ephippium*) by day 130 post hatched. The transition from juvenile to adult body coloration in the hybrid clownfish was achieved within 130 days post hatched, much sooner than its parent species, *A. ephippium*, which takes 310 days to transform completely. The hybrid clownfish's final body pattern is unique; it consists of a white band near the head (resembling one of the parent species, *A. melanopus*) and a black blotch at the side (resembling the other parent species, *A. ephippium*).

The length of the hybrid larvae showed a substantial increase from day 20 post-hatch (8.60 ± 0.34 mm) to 130 days post-hatch (34.30 ± 1.63 mm) (Figure 4). Compared to its parent species, *A. ephippium*, the growth in the size of the larvae of the hybrid clownfish was much bigger and faster. *Amphiprion ephippium* is reported (Rohini Krishna et al., 2018) to reach its maximum length of 22.62 mm in 6 months, while the hybrid clownfish almost reached its maximum length of 34.30 mm in 130 days post hatched. In 6 months after hatching,

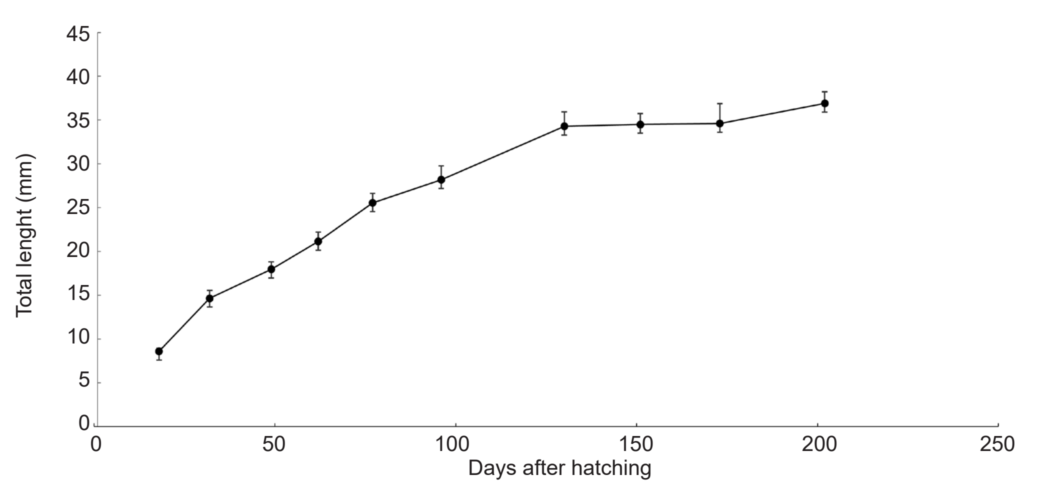

Figure 4. Total length (mm) of hybrid larvae (*Amphiprion ephippium*♂ × *Amphiprion melanopus*♀) post-hatching

the hybrid clownfish juveniles reached the maximum length of 36.90 ±1.32 mm, almost 14 mm larger than its parent species, *A. ephippium*. Therefore, the hybrid clownfish (*Amphiprion ephippium* ♂ × *Amphiprion melanopus* ♀) is a suitable candidate as a marine ornamental fish culture for the ornamental trade due to its unique body coloration and pattern that it inherited from both its parent species.

CONCLUSION

Compared to its parent species, *A. ephippium*, the hybrid clownfish (*Amphiprion ephippium* ♂ × *Amphiprion melanopus* ♀) larvae underwent metamorphosis earlier and reached the marketable size much faster. The body coloration is also unique, where the features from both parent species were inherited and expressed. The most remarkable finding in this study is that a clownfish can establish a sexual relationship with other clownfish species, and their offspring can indeed be successfully reared in captivity.

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