A review of the reproduction and rearing of clownfish, *Amphiprion clarkii*, in captivity

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Abstract

Clownfishes are very popular among ornamental fish keepers due to their aesthetic appeal and easy adaptability to captive conditions. The captive breeding of these fish not only meets the market demand but also prevents the destruction of their natural habitats during fishing. In Iran, *Amphiprion clarkii* has been successfully bred and reared in the Bandar Lengeh mollusks research center. The broodfish were fed twice a day in the morning and the afternoon at 5% of body weight. The pot was used as a shelter for broodfish spawning in the aquarium. One indication of impending spawning was the male and female broodfish cleaning inside the shelter. In this species, the number of eggs per spawning was 300-400. The eggs were sticky and protected by their parents during the incubation period. At 28 ◦C, the eggs were hatched after six days. During the first two weeks after hatching, rotifer and Artemia nauplii were exclusively utilized to feed the larvae. The weaning process started 15 days after hatching (DAH), and on 25 DAH, all the larvae completely shifted to the microparticulate diet. To improve the color of farmed fishes, the formulated feed prepared for the larvae and juveniles was supplemented with 100 ppm and 200 ppm astaxanthin, respectively. During 2-4 months, the juveniles reached the size of 3-4 cm and were ready to be sold in the market. The survival rate during this period was more than 50%.

Keywords: Ornamental fish, Salt water, Clownfish, Breeding and rearing, Larval and juvenile

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Introduction

Marine ornamental fish trade is a rapidly expanding industry, to the extent that the value of reef fish exports to various countries reaches 300 million dollars worldwide (Palmtag, 2017). The primary focus of this trade is in tropical regions worldwide, particularly Southeast Asia, serving as the main export hub for this product to the US and Europe. Damselfish (Pomacentridae) constitute nearly half of this trade, while other species like Pomacanthidae, Acanthuridae, Chaetodontidae, and Gobidae make up another 25-30% (Olivotto *et al.*, 2009). While the trade of marine ornamental fish is increasing rapidly, there is also resistance towards purchasing wild-caught fish due to the growing environmental awareness among people and the concern for the extinction risk facing these creatures. Consequently, this issue has amplified the demand for farm-reared fish and provided an attractive aquatic commercial development (Olivotto *et al*., 2003). However, despite the many efforts made in recent years to develop the aquaculture of marine ornamental fish, this industry is still in the early stages of its development compared to the aquaculture of other fishes. For instance, the number of marine ornamental fish bred in captivity is much lower than that of freshwater ornamental fish. Indeed, about 98% of all species sold in the marine ornamental fish industry have been caught from their natural habitats (Pouil *et al*., 2020).

Among marine ornamental species, clownfish are the most popular in the aquarium trade. They are popular as aquarium fish for their bright colors, small size, and remarkable hardiness. There are 225 species in the family Pomacentridae in approximately 25 genera, mostly natives of Indo-Pacific reefs (Olivotto *et al*., 2009). Two species of this family, including *Amphiprion clarkii* and *Amphiprion seba*, are inhabitants of the Persian Gulf and Oman Sea, with considerable commercial value in Europe and the USA. They can be looked upon as appealing candidates for marine ornamental aquaculture. On the other hand, there are also good potentials for successful captive spawning and cultivation of these two species on commercial scales for exporting to target markets overseas, where economic equations signify trading with the Middle East is more economical due to its shorter distance compared to South East Asia.

As with other marine fish species, the major bottleneck in this species' reproduction and rearing process is related to the larval stage (Kolkovski, 2013). Therefore, in breeding and rearing marine ornamental fish, designing an accurate method to meet the nutritional requirements of larvae, particularly from the onset of exogenous feeding to the metamorphosis stage, is crucial. Hence, a significant portion of studies on the reproduction and rearing of this species should be dedicated to this issue. In Iran, at the Bandar Langeh mollusk research center, the species *Amphiprion clarkii* has been successfully bred and reared on a large

scale. The current study aimed to review the methods employed at this center for broodstock development and their reproduction and the rearing of this species' larvae and growing them to the market size. Transferring the technical knowledge of the reproduction and rearing of this species to the private sector and increasing its production can be a prelude to their entry into the profitable industry of marine ornamental aquaculture in domestic and international markets. Also, for a better understanding of the breeding process of marine ornamental fishes, the design of a small-scale hatchery was briefly described for beginners.

Difference between clownfish larvae and other marine fish larvae

A major difference between clownfish larvae and other marine fish larvae stems from their adult spawning strategies. In fact, as opposed to other marine fish, which release millions of small pelagic eggs in the water body without protecting them against predators, clownfish lays a limited number of eggs in a shelter, and then guard them until they hatch.

Altricial larvae, upon hatching, possess a rudimentary digestive system, and the differentiation of their digestive system and the formation of the stomach are postponed until metamorphosis. These larvae are hatched from small eggs and possess an embryonic period with inferior swimming ability. Then, during the subsequent larval stage, they gradually undergo further development (Chen *et al*., 2020). Therefore, the altricial larvae rely on live food for feeding during the initial weeks after hatching. Conversely, precocial larvae hatch from larger eggs and have a more developed digestive system at the time of hatching, and this enables them to easily feed on formulated diets as soon as they hatch from the eggs (e.g., Salmonidae) (Pan *et al*., 2022). Although clownfish larvae are not considered precocial species, they are classified somewhere between altricial, and precocial larvae (Gordon and Hecht, 2002; Onal *et al*., 2008). Histological findings indicate that clownfish larvae have a relatively developed digestive system at the time of hatching. In particular, compared to species with altricial larvae, the development of gastric glands occurs earlier in clownfish larvae (between 11 to 15 DAH) (Onal *et al*., 2008). For example, the formation of gastric glands in sole and European seabass larvae occurred at 22 and 25 DAH, respectively (Zambonino Infante *et al*., 2008). In contrast, in the gilthead seabream larvae, this phenomenon was postponed to the sixth week after hatching (Elbal *et al*., 2004). The development of gastric glands in fish larvae indicates the end of metamorphosis and the emergence of a functional stomach (Zambonino Infante *et al*., 2008; Kolkovski *et al.*, 2009). The development of gastric glands enables the larvae to digest various food items. This issue facilitates the reduction of larval production costs since, at this stage, the possibility of substituting live food with a formulated diet becomes available. Therefore, compared to

altricial larvae of marine fishes, this represents a significant advantage for the aquaculture of different species within this family under captive conditions. In general, live food makes up 79% of the production cost for juveniles up to 45 days after hatching. Moreover, in the initial three months of life, live food accounted for 50% of the feed cost, although it comprised just 1.6% of the total dry weight of required food (Kolkovski, 2013).

Another positive point of clownfish larvae compared with other marine larvae lies in their suitable mouth-gape size when they hatch out (250 µm). Whereas, generally, marine food fish larvae have a closed mouth at the time of hatching, and a small mouth-gape size at the commencement of exogenous feeding (Yufera and Darias, 2007). The small mouth size of the larvae at the time of hatching is one of the main reasons for limiting the initiation of exogenous feeding (Yufera and Darias, 2007). As a result, the protocol for rearing marine food fish larvae with small mouth gaps involves feeding newly hatched larvae with small rotifers. This is achieved by passing a mixed rotifer culture through a fine mesh screen (90 µm plankton net) (Marte, 2003). However, clownfish larvae do not require specially selected live food such as sieved rotifers or other tiny plankton from their first feeding.

Broodstock pair formation

Fish were purchased from a pet shop. The *A. clarkii* mature into a spawning pair when they are approximately one year old (measuring 5-10 cm) (Olivotto *et al*., 2009). Pair formation was accomplished in a large mono-species community of 200-L rectangular-shaped glass tanks, which contain plenty of refuges. If the tank conditions are suitable, the fish in the tank reach maturity and begin displaying mate selection behavior. In this condition, the dominant large female in the tank constantly harasses the smaller fish until it acquires the male characteristics and then goes to the shelter with the female.

Broodstock conditioning

Once pair formation occurred, the new pair was removed from the community tank to an isolated broodstock tank In order to get fish to spawn, water quality in the tank needs to be stable. In other words, the physicochemical conditions in the tank have to be consistent to mimic the natural environmental conditions (Table 1).

Table 1: The Physicochemical condition in the broodstock tanks.

Tank volume (L)	Temperature (˚C)	Photoperiod	pH	Salinity (ppt)	O_2 (ppm)	NH_3 (mg/L)	Light intensity (lux)
200	27-28	14L/10D	7-8	42-43	>7	<0.1	600-700

All clownfish tanks were equipped with blue nightlights. Young fish often eat their egg clutches, but this does not occur when a nightlight is set up (Olivotto *et al*., 2011). In the wild, clownfish establish and protect a small territory close to an anemone, and exhibit similar behavior in captivity. Unfortunately, anemones do not survive for long periods in captivity, and clownfish usually accept other materials as a shelter. For instance, anemone can be entirely substituted by 10-15 cm diameter flowerpots (Olivotto *et al*., 2008; Olivotto *et al*., 2009). Therefore, several flowerpots were placed in the broodstock tank to allow the pair to select the most suitable substrate for laying eggs. Proper nutrition of broodfish not only improves their health but also affects the quality of gametes. Therefore, the broodfish were fed twice a day, in the morning and early evening, with a prepared wet diet (Table 2).

Table 2: The composition of the wet (g/100g).

Ingredients	Value
Fresh Fish	26%
Fresh Squid	26%
Fresh Shrimp	26%
Gelatine	10%
Vitamin C	3%
Vitamin E	2%
Vitamin premix	2%
Mineral premix	2%
The mixture of fresh lettuce and spinach leaves	3%

The wet diet consisted of a combination of fresh fish, squid, and shrimp in an equal ratio, which was finely chopped along with spinach or lettuce leaves in a blender. Ascorbic acid polyphosphate and alpha-tocopherol acetate were utilized as sources of vitamins C and E, respectively. After all components were well mixed in a mixer, the food was bound by adding a 10% gelatine mass (dissolved already in tap water) to the mixture. The food was then divided into small portions and frozen until use. Fish were fed at 5% body weight per feeding. Due to the fact that in nature, clownfish feed on approximately 36% plant-based resources, every day, a few fresh lettuce leaves were placed in the spawning tanks (Tartila *et al*., 2023).

Egg collection and incubation

If suitable husbandry and appropriate environmental conditions were provided, clownfish would easily spawn in captivity. The *A. clarkii* pairs always spawn in the afternoon, and their egg clutch contains about 300-400 eggs (Olivotto *et al*., 2009). At 28°C, hatching took place eight days after fertilization. The eggs need to be removed from the spawning tanks before hatching; otherwise, collecting the larvae after hatching can cause damage to them, and also, the parents will feed on them.

Embryos change their color dramatically from orange to grey, then black during development (Tartila *et al*., 2023). The day before hatching, the eggs are silver in color, and the larvae's eyes can be observed inside the egg with metallic blue or green pigment. Thus, eye development may be an excellent marker to follow embryo development and predict hatching time. The evening that hatching was predicted, the flowerpot with egg clutch was transferred to a larval-rearing tank. The

eggs must be kept submerged in water at all times. The physicochemical conditions of the two tanks must be identical since any minor variation can cause a hatching delay (Olivotto *et al*., 2009). In larvae-rearing tanks, parental care can be replaced by positioning an air stone under the egg clutch. The airflow was adjusted to obtain a delicate flow that gently moved and oxygenated the embryo. At this time, the lights turned off. Hatching occurred about 45 to 60 minutes after lights out (Green *et al*., 2006). If the eggs did not hatch after two hours of darkness, they returned to the broodstock tank under the darkness cover.

Larval rearing

The hatching day was called day 0. The larvae were left in darkness until the next morning. The larvae were raised in 200-L round tanks with black walls and white bottoms. *Nannochloropsis oculata* (50000 cells/mL) was introduced into the tanks from the day after hatching until nine days later. The microalgae density was kept constant by assessing cell density twice daily under a light microscope. Algal cells in 1 mL samples were counted using a Nenbauer hemocytometer, and the results were averaged. Larvae were raised in an open system with no water exchange on day 0, and from 1 DAH until the end of the project, 50-200% of water volume was replaced daily. Ultraviolet-filtered seawater, which had been filtered through a sand filter, was utilized for larval rearing. The clownfish usually begin feeding within 8 hours after hatching. Therefore, from 1 DAH, a density of ten enriched rotifers/mL (*Brachinous plicatilis*) was supplied in larval-rearing tanks. The rotifer density was reduced to five rotifers/mL by 7 DAH, and on 10 DAH, rotifers were withdrawn from larval rearing tanks. From 7 DAH enriched *Artemia* nauplii was initially introduced to the larvae rearing tanks at a concentration of two nauplii/mL (Olivotto *et al*., 2009). From 9 to 15 DAH, this number was gradually increased to six nauplii/mL. Then, from 16 DAH up to 25 DAH, the density of *Artemia* reduced to two nauplii/mL, and at 26 DAH, they pulled out of the tanks. The density of rotifer and Artemia in the larval-rearing tanks was adjusted twice a day (morning and afternoon). To maintain the optimal live food density in the tanks, three samples of 20 mL were taken from different parts of the tank before each meal. The number of live food organisms in each sample was counted under a light microscope, and any deficiencies were compensated by adding fresh prey items. The clownfish larvae started to exhibit initial signs of metamorphosis on 9-11 DAH (Tartila *et al.*, 2023)., and this process was completed by 25 DAH (Onal *et al*., 2008). Therefore, it is possible to introduce an artificial diet to larval-rearing tanks with no reduction in their survival rate from 15 DAH onwards. In the current project, clownfish larvae were fed on a combination of *Artemia* nauplii and a prepared microparticulate diet from 15 DAH, and the larvae were fully weaned onto the artificial diet on 25 DAH. The

composition of the larval weaning diet in this project is detailed in Table 3. In this diet, ascorbic acid polyphosphate and alpha-tocopherol acetate are used as sources of vitamins C and E, respectively. In addition, squid meal was incorporated into the diet as part of the protein source in order to improve the diet's palatability due to its attraction function.

Table 3: The composition of the larval weaning diet (g/100g)

Ingredients	Value
Fish meal	45%
Squid meal	25%
Fish oil	10%
Soy lecithin	5%
Carrageenan	6%
Vitamin C	2%
Vitamin E	2%
Vitamin premix	2.99%
Mineral premix	2%
Astaxanthin	0.01%

Furthermore, the diet was supplemented with 100 ppm astaxanthin to regulate the skin color and pigmentation of *A. clarkii* larvae (Yasir and Qin, 2010). Carrageenan was utilized in the diet as a binder as well. The diet was prepared using the microbound method (Langdon, 2003) as follows: After mixing the components of the microdiet, an aqueous solution containing 6% carrageenan was added as a binder. The mixture was well mixed until a uniform paste was obtained. The paste was poured on aluminum pans and placed inside an oven at 45°C for 72 hours (Lian *et al*., 2008). The dry diets were then grounded and sieved to the required size shown in Figure 1 (250, 350, and 500 to 800 µm). The larval rearing room was on a 14L:10D photoperiod cycle (Chen *et al*., 2020). To minimize bacterial, fungal, and pathogenic growth, sediments in the larval-rearing tanks were siphoned out just before lights were out, as displayed in Figure 1. The room was brightly lit by biolux fluorescent tubes mounted on the ceiling, accompanied by 100-watt incandescent bulbs suspended 1.0 meters above the water surface of each larval-rearing tank. The light intensity at the water surface ranged between 800 and 900 lux, as reported by Dhaneesh *et al*. (2012). Additionally, a surface skimmer constructed from PVC piping was employed to eliminate the oil film from the water surface during the initial two weeks of the larvae's development (Fig. 1). The post larvae were kept in larvae-rearing tanks until they became sufficiently strong, and then as they grew larger (15-20 mm), they were moved from the larvae-rearing room to grow-out tanks.

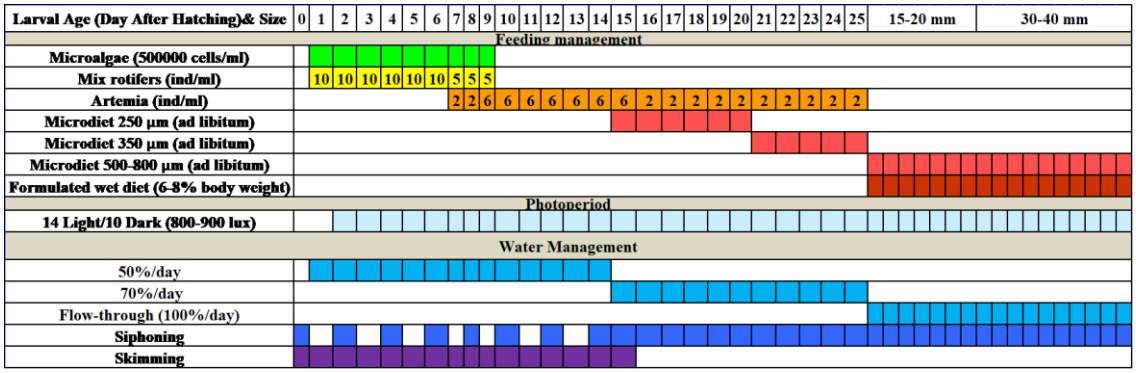

Figure 1: The *A. clarkii* larvae rearing protocol.

Juvenile grow-out

The juveniles were introduced into grow-out tanks at a stocking density of one fish per liter. They were fed on the prepared microdiet (500-800 µm) ad libitum in the complete absence of live prey from 26 DAH onwards. To improve juvenile grow-out performance, the dry diet was supplemented with a developed wet diet (Table 4) twice a day at 6-8% of juvenile body weight (Fig. 1). This diet was prepared according to the instructions provided already for the preparation of wet diet used to feed broodfish.

Table 4: The composition of the grow-out wet diet (g/100g)

Ingredients	Value
Fresh fish	64%
Fresh chicken heart	18%
Gelatin	10%
Vitamin C	2%
Vitamin E	2%
Vitamin premix	1.98%
Mineral premix	2%
Astaxanthin	0.02%

At this stage, dark green tanks (600-L) were used to grow the juveniles. Research has documented that *A. clarkii* juveniles raised in vessels with dark backgrounds exhibit higher survival rates compared to those reared in vessels with light backgrounds (Tartila *et al*., 2023). In addition, it has been reported that tanks with dark backgrounds enhanced the red-orange color of skin in some of this family species, such as *Amphiprion ocellaris* (Yaisr and Qin, 2009). The clownfish typically require 2 to 4 months to attain market size, which is 30-40 mm in total length (Olivotto *et al*., 2009).

Generally, fish reared under captivity conditions tend to have less vibrant colors compared to wild-caught fish. The main reason for this issue is the lack of access of farmed fish to natural sources of carotenoids. Therefore, carotenoids such as astaxanthin must be provided to juveniles through food sources used to feed them to maintain their bright color in addition to other duties like functions in the immune system of the creature. Consequently, 200 ppm astaxanthin was added to the diet in order to improve the livability of the raised juvenile.

Enrichment of live prey

As mentioned earlier, in the current project, live feeds (rotifer and *Artemia*)

were enriched before adding them to larvae-rearing tanks.

Techniques for protein enrichment

Rotifers naturally do not contain sufficient protein to meet the nutritional requirements of larvae (Kandathil Radhakrishnan *et al*., 2020). However, feeding rotifers with protein-rich sources can indeed increase their protein content. The most common alternative food source to substitute algae in the cultivation of rotifer is baker's yeast. Algae are the best source of unsaturated fatty acids, while baker's yeast contains significant amounts of protein. For this reason, these two food sources are used combined for culturing rotifers. In the batch culture technique, the rotifer rearing tanks are half-filled with algae at a density of $13\text{-}40\times10^6$ cells/mL and inoculated with rotifers at a density of 100 individuals/mL. On the first day, baker's yeast is added to the culture tanks twice a day at 0.25 grams per million rotifers. The following day, the tanks are fully filled with algae at the same density, and the baker's yeast is used at 0.37 grams per million rotifers twice a day. The next day, the rotifers are harvested, and new tanks are inoculated (Kandathil Radhakrishnan *et al*., 2020).

Techniques for (n-3) HUFA, vitamin C and E enrichment

One of the common enrichment mediums used to boost rotifer and Artemia is oil emulsion that is rich in polyunsaturated fatty acids and phospholipids, leading to an improvement in their EPA, DHA, and ARA content. In summary, a combination of distilled water (380 mL), cod liver oil (400 mL), and soy lecithin (200 mL) was used to make an oil emulsion (1000 mL). The mixing process involved heating the cod liver oil and soy lecithin to 50°C and then adding them to the preheated distilled water at the same temperature. An emulsifier, such as Polysorbate 80 (Tween 80), was added to this oil-based mixture (20 mL). Then, the mixture was placed in a blender and mixed until a cream-colored mixture was achieved (Mutti, *et al*., 2017). In order to increase the vitamin C and E content of both rotifer and *Artemia*, 20% (w/w) ascorbyl palmitate and alpha-tocopheryl acetate were added to the booster to supplement it with vitamin C and E as well (Singh *et al*., 2023). The emulsified oil was then transferred to a darkish bottle and stored at 5°C.

Rotifer and *Artemia* were enriched with the booster as micro follows: The rotifers were harvested using a 50 µm net. In a 30-liter container filled with filtered seawater, rotifers at a density of 100 rotifers/mL were introduced along with an oil emulsion at a concentration of 1 mL/L. After 12 hours, the rotifers were filtered, washed several times with fresh seawater, and then utilized as feed for the larvae. Newly hatched Artemia nauplii were separated using a 60 µm mesh. The Artemia nauplii, with a density of 15 nauplii/mL, were added to a 50-liter bucket containing filtered seawater. The oil emulsion with a concentration of 1 mL/liter was poured into the bucket as well. After 12 hours,

the nauplii were filtered and washed multiple times to remove excess oil, and then fed to the larvae (Pham *et al*., 2023).

Hatchery design

In this section, the design of a small-scale marine ornamental hatchery is described briefly. For easier temperature control and cost reduction in construction, it is better to design one unit for a marine ornamental fish hatchery. In other words, different sections of the hatchery, including broodstock, larval rearing, grow-out, live food, and packaging rooms, are all located under one roof with separating walls (Fig. 2). This system not only helps to reduce energy consumption but also assists in controlling diseases outbreak among different sections. A compromise is to centralize all systems under one roof while segregating them into individual rooms within the hatchery that can be effectively isolated from one another. The brood stock system stands out as the most critical component. While juvenile fish can be readily replaced, any loss of broodstock fish or decline in egg production would have a disastrous impact on hatchery production. The hatchery must include a dedicated quarantine room, which should be entirely isolated from other sections of the facility. Even if the quarantine room shares a common structure with other areas of the hatchery, it must be fully sealed off and have a separate entrance exclusively for entry and exit, accessible from outside the hatchery. The basic hatchery layout with various sections is displayed in Figure 2.

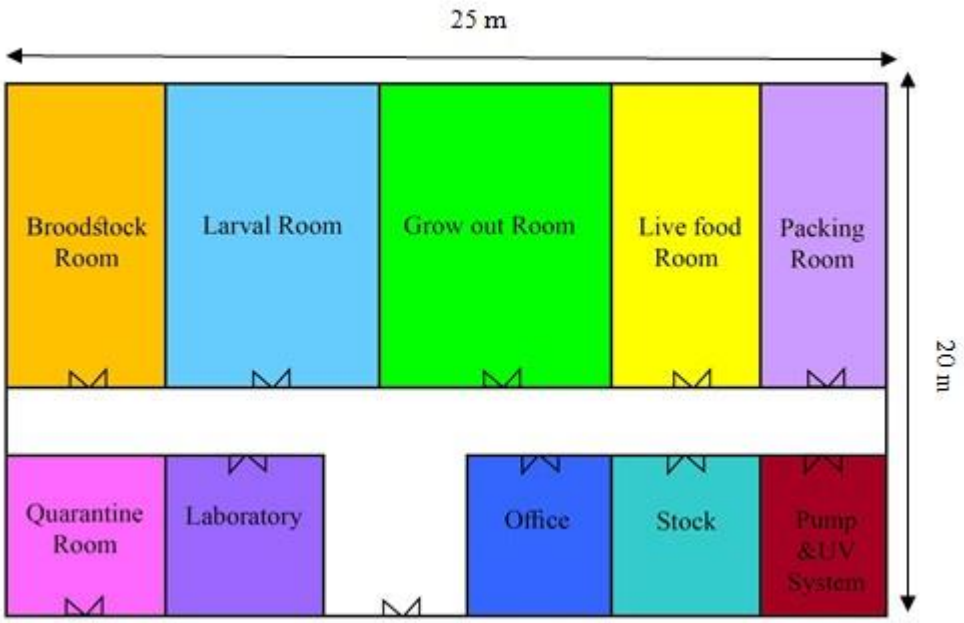

Figure 2: An example of a marine ornamental fish hatchery in one insulated building.

As depicted, each section features an entrance accessible from the central corridor, eliminating the need to pass through one room to access another. Every room must be equipped with provisions for electricity, saltwater, and

freshwater. Furthermore, each section should be outfitted with its dedicated equipment, including buckets, nets, siphoning hoses, and other necessary tools. It's imperative that this equipment is never shared between different sections to maintain biosecurity and prevent cross-contamination. Also, in a marine aquarium hatchery, some crucial points should be taken into consideration:

1. Since humidity in a well-insulated hatchery will quickly reach saturation point, the hatchery must have a powerful exhaust fan. Wind-driven roof ventilators use only environmental resources to swap out hot and are very cost-effective.
2. The hatchery utilizes air conditioning to maintain a consistent indoor ambient temperature range of 21-25°C.
3. Immersion thermal heaters should be employed in each hatchery section to adjust the water temperature to the optimal level depending on the cultured species.
4. To mitigate the risk of disease outbreaks, maintaining cleanliness in the hatchery is paramount. To facilitate this, the floors should be tiled and the walls should be covered with ceramic. These surfaces enable easy disinfection and washing, helping to uphold stringent hygiene standards.

Conclusion

Fortunately, Iran's southern coastal habitats are home to two species, *Amphiprion sebae* and *Amphiprion clarkii*, belonging to the subfamily Amphiprioninae. This natural occurrence suggests that Iran holds promising potential for the breeding and rearing of these species. Considering that in recent years, a significant portion of fish species within this subfamily has been exported from Southwest Asia to Europe and America, it is, therefore, possible to meet some of this demand through Iran by mass breeding of these fish and addressing existing export barriers. Despite facing stiff competition with Southeast Asian countries in this field, achieving success is feasible for Iran. One contributing factor to this feasibility is Iran's geographical proximity to Europe compared to Southeast Asian nations. This geographical advantage potentially facilitates the transportation and export of marine ornamental fish to European markets from Iran. It appears that gaining access to markets of the countries of Central Asia, Azerbaijan, and Iraq, which lack natural reserves of marine ornamental fish, can suffice for boosting exports of these species from Iran to these countries and ultimately yield profits for the private sector.

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