# A star is born again: Methods for larval rearing of an emerging model organism, the False clownfish Amphiprion ocellaris

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RESOURCE ARTICLE

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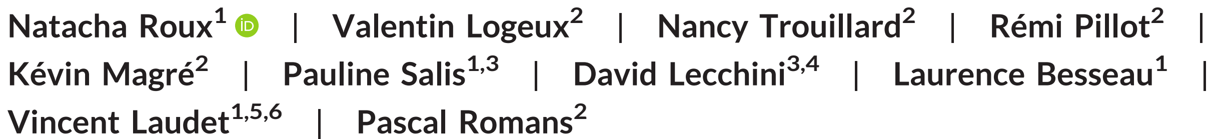
# A star is born again: Methods for larval rearing of an emerging model organism, the False clownfish *Amphiprion ocellaris*



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## Abstract

As interest increases in ecological, evolutionary, and developmental biology (Eco-Evo-Devo), wild species are increasingly used as experimental models. However, we are still lacking a suitable model for marine fish species, as well as coral reef fishes that can be reared at laboratory scales. Extensive knowledge of the life cycle of anemonefishes, and the peculiarities of their biology, make them relevant marine fish models for developmental biology, ecology, and evolutionary sciences. Here, we present standard methods to maintain breeding pairs of the anemonefish *Amphiprion ocellaris* in captivity, obtain regular good quality spawning, and protocols to ensure larval survival throughout rearing. We provide a detailed description of the anemonefish husbandry system and life prey culturing protocols. Finally, a "low-volume" rearing protocol useful for the pharmacological treatment of larvae is presented. Such methods are important as strict requirements for large volumes in rearing tanks often inhibit continuous treatments with expensive or rare compounds.

KEYWORDS

*Amphiprion ocellaris*, anemonefishes, coral reef fish, eco-evo-devoexperimental model, rearing methods

## 1 | INTRODUCTION

In developmental biology, few species (among which the mouse *Mus musculus*, the chicken *Gallus gallus*, the xenopus *Xenopus laevis*, the fruit fly *Drosophila melanogaster*, the worm *Caenorhabditis elegans*, and the zebrafish *Danio rerio*) are the most common experimental models. Usually called "the big six" (Jenner & Wills, 2007), these models have been selected due to the simplicity of keeping them under laboratory conditions, and the possibility they offer to produce mutants and develop complex genetic tools. Although these species have facilitated great scientific breakthroughs in multiple domains such as genetics, developmental biology, medicine, and so forth, they

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represent only a tiny fraction of living biodiversity. For this reason, new model species have emerged in Evo-Devo in the past few years (e.g., the amphioxus *Branchiostoma lanceolatum*, Bertrand & Escriva, 2011, the sea squirt *Ciona intestinalis*, Corbo et al., 2001), the annelid worm *Platynereis dumerilii* (Fischer & Dorresteijn, 2004), the sea anemone *Nematostella vectensis* (Darling et al., 2005). Moreover, several teleost species such as medaka, cavefish, or stickleback (Cresko et al., 2006; Jeffery, 2008; Merilä, 2013; Wittbrodt et al., 2002) have proven their potential as model species for tackling Eco-Evo-Devo questions. These new models have allowed for a more global outlook on the complexity of living systems and evolutionary processes in a developmental context. However, we are still lacking marine teleosts that are suitable model organisms for the Eco-Evo-Devo investigations.

Marine teleost species used for research purposes are primarily the aquaculture models (sea bass, sea bream, flatfish, tuna, and grouper). Unfortunately, the size of adult individuals, difficulties in their sex identification, and their low spawning frequency make these species unfit to maintain and reproduce at laboratory scales (i.e, aggressive behavior). Nevertheless, a group of marine fishes has received special attention over the past decade: The anemonefishes. The potentialities of anemonefishes as models for Eco-Evo-Devo have been recently reviewed (Roux et al., 2020). Their remarkable biological traits such as sex changes, symbiotic relationships with sea anemones, social organization, and sound production, have meant they have been the subject of many studies in recent years. Such studies have addressed scientific questions spanning multiple disciplines such as ecology, developmental biology, and evolutionary sciences (Abdullah & Saad, 2015; Buston, 2003; Dixson et al., 2014; Litsios et al., 2012; Marcionetti et al., 2019; Mebs, 2009; Sahm et al., 2019). With the potential for use in behavioral experiments that can be performed in the lab or directly in the wild, or in functional genetics and genomic studies, these fishes undoubtedly provide an attractive marine model for Eco-Evo-Devo as well as for ecotoxicological studies (reviewed in Roux et al., 2020). Toxicological studies are primarily concerned with investigating the effects of various chemicals and their compounds on organisms or conducting functional studies to enhance our understanding of a biological process. For example, teleosts treated with compounds that block thyroid hormone synthesis or action, slow down metamorphosis and help to better understand the role of TH in metamorphosis (Campinho et al., 2018; Holzer et al., 2017). Moreover, as inhabitants of coral reefs, they will provide fish models to study these ecosystems that are increasingly threatened by global climate change.

Considering this, our group contributed to establish the False clownfish *Amphiprion ocellaris* as an experimental model species in Eco-Evo-Devo by describing its larval development (metamorphosis process) and the ontogeny and evolution of its pigmentation pattern (Roux et al., 2019; Salis et al., 2019; Salis, Roux, Soulat, et al., 2018). *A. ocellaris* larval development has been characterized using morphological criteria (such as notochord flexion, fin ray appearance, white bars formation) into seven distinct stages (Roux et al., 2019). Briefly, stage 1 corresponds to preflexion larvae without differentiating fins, and a pigmentation pattern consisting of black melanophores over yellow xanthophores spreading from the head to the caudal peduncle. At stage 2, the notochord is in flexion and enters into postflexion at stage 3. Fins start to develop in these two stages, with rays growing. Pigmentation patterns remain similar to stage 1. At stage 4, orange xanthophores appear, dorsal and ventral fin rays are fully developed, and pelvic spines are growing. Furthermore, the overall shape of the larva becomes more ovoid. Transparent white bars on the head and body start to appear at stage 5 and become whiter at stage 6. Stage 7 is characterized by the emergence of the third white bar on the caudal peduncle, and finally, the juvenile stage is reached when fin pigmentation is complete.

As for all anemonefishes, *A. ocellaris* is a monogamous protandrous hermaphrodite (male to female transition) that lives in groups and in symbiosis with sea anemones (Fautin & Allen, 1994). This group consists of a breeding pair and sexually immature juveniles ranked by size (Buston, 2003). The breeding pair, composed of a large dominant female and a smaller subdominant male, is established through aggressive behavior. The most aggressive and dominant individual will become a reproductive female and the second will turn into a reproductive male. This dominant pair prevents the sexual maturation of subdominant juveniles. Death (or removal) of the dominant female induces the sex inversion of the dominant male and thus allows the biggest immature juvenile to become the reproductive male (Casas et al., 2016). Due to their ability to reproduce in aquaria, their spawning frequency (every 2–3 weeks), the ease of collecting large quantities of eggs laid on a substrate (100–500 eggs), and their short embryonic and larval development (respectively, 10 and 15 days at 26°C), *A. ocellaris* has all the qualities required to be reared in laboratory conditions and used for experimental purposes. Noteworthy is that anemonefishes in aquaria do not require the presence of a sea anemone making their maintenance easier. Indeed, the role of the sea anemone is to provide shelter and to help avoid predators only.

Although rearing techniques for anemonefishes were previously well documented (Divya et al., 2011; Kumar et al., 2012; Madhu et al., 2006), there were no established methods to perform pharmacological treatments in low volumes. Being able to rear anemonefish larvae in small volumes (less than 1 L) is critically important, as rearing volume can be an issue for pharmacological treatments that last several days, especially when the compound is either rare (e.g., synthesized in small amounts), toxic (considering the volumes of seawater to be treated), and/or expensive. Thus, the description of low-volume protocols for anemonefish rearing is valuable, as to our knowledge, such information is not available in the literature for any other marine fish.

We present methods that we have developed to rear *A. ocellaris* larvae in a calibrated system. We describe the anemonefish husbandry system, methods to maintain breeding pairs and obtain regular spawning, and to culture the live prey required for larval rearing. Finally, detailed methods for rearing anemonefish larvae in classical conditions (in 54-L tanks filled with 30 L of seawater) as well as in low-volume conditions (in 500-ml glass beakers) are provided. The

methods described will enhance the range of experimental possibilities offered by this promising marine fish model and may be adapted for other marine species.

## 2 | ANEMONEFISH HUSBANDRY

### 2.1 | Technical system

Adult anemonefish can be maintained in rearing structures similar to those developed for amphioxus or zebrafish, but with some adaptations (Carvalho et al., 2017; Lawrence & Mason, 2012). The husbandry system described in the present paper has been set up using medium-sized heavy shelving (300 × 240 × 60 cm) divided into three levels. The system is operated with a recirculation system of artificial seawater (Red Sea salt, Antinéa) and is composed of 30 individual tanks (60 × 30 × 35 cm, 63 L, Antinéa), 10 per level (Figure 1). The first level is dedicated to growing juveniles, and the second and third levels are for breeding pairs. Inside each tank, there is a vertical outlet PVC pipe equipped with a mesh (to prevent fish from escaping into the filtration system) that facilitates the flow of seawater (via gravity) into an 800-L technical sump tank below the rearing tanks (Figure 1). This technical tank is equipped with filtering foams, a phosphate reactor (500 L/h Deltec FR512, Poisson d'Or) filled with 1 L of resin (ROWA Phos), a skimmer (2 × 1260 H&S; Le Monde de la Mer), a UV sterilizer (UV-C JBL 2 × 36 W; Antinéa), and a lift pump which allows the return of filtered seawater to each tank through an inlet PVC pipe at the rate of 300 L/h (11,500 L/h, Vectra L1, Ecotech Marine). Each inlet pipe is equipped with a tap to regulate water flow. The technical tank is also filled with live rocks (100 kg) to ensure the biological filtration of the rearing structure. All these elements maintain low levels of nitrogen compounds and phosphates, which is essential for successful anemonefish reproduction and ensuring high hatchling survival rates (Callan, 2007; Edwards et al., 2005). A third of the total volume of the husbandry system is monthly replaced by new artificial seawater to maintain the water quality. Temperature is kept constant in all tanks by two titanium heaters (2 × 500 W, Aqua Medic; Poisson d'Or) located in the technical tank and connected to a temperature controller (Aqua Medic T-controller twin; Poisson d'Or). To avoid any variation in the developmental timing of the fish, the tank temperature has to be constant. In our case, we added an air conditioning system to the room that can be used in summer if the ambient temperature increases too much in the room and also in the tank. Light is provided by white/blue LED lamps, fixed above each level of tanks (Lumivie, 50 W, 150 cm; Poisson d'Or). The photoperiod is controlled by a light controller (Aquavie Varioled V2, Poisson d'Or).

### 2.2 | Breeding pair maintenance

Multiple conditions must be met to ensure optimal spawning in terms of frequency and quality. First, the husbandry system has to be in a quiet room, in which the movement and entry of curious by-passers should be limited to reduce breeding pair stress. Second, it is important to place each breeding pair in separate tanks and to ensure they do not see their neighbors or their reflection in the glass tank walls. This constrains aggression disrupting reproductive behavior. It is, thus, advised to separate each tank with white panels and allow algae to colonize tank walls to avoid mirror effects. An additional

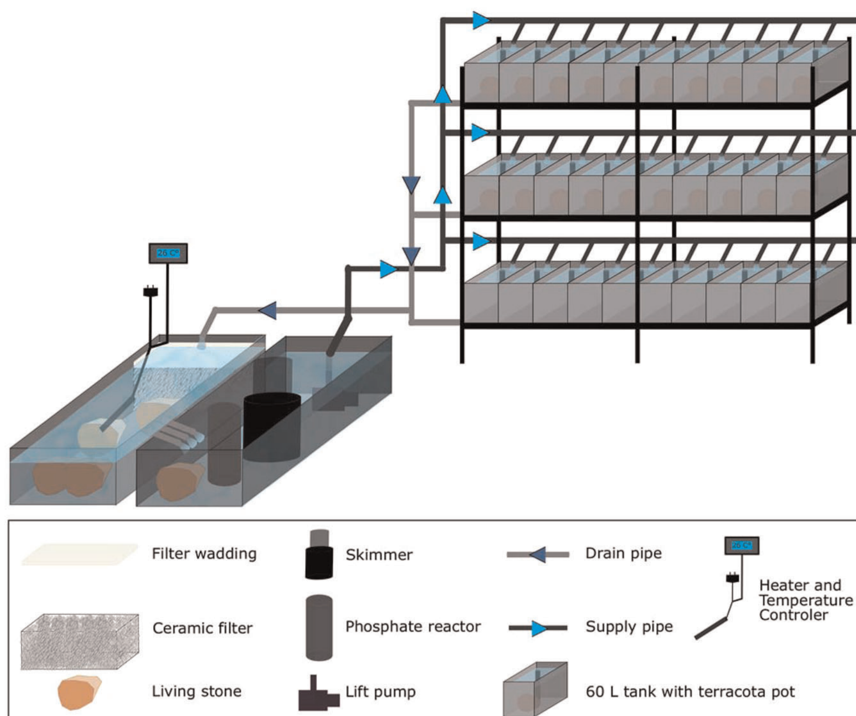



**FIGURE 1** Schematic diagram of the anemonefish husbandry system in the Banyuls-sur-mer laboratory. This illustrates the general organization of the husbandry system, with the juvenile growing tanks on the first level and the breeding pair tanks on the second and third levels of the husbandry system. The technical tank with the filtration system is also illustrated

benefit of this is that algal development has a positive effect on water quality.

Breeding pairs can be directly purchased from pet shops or obtained by selecting a big and smaller individual from a group of sexually immature juveniles to form a new pair. It is worth noting, that it will take between 6 months and 2 years for the newly formed pair to reproduce for the first time. Third, for anemonefish to reproduce, it is critical to closely monitor water parameters such as temperature (26–28°C), salinity (30–35 g/L), and pH (8–8.5) while also maintaining a stable photoperiod (12 of 12, 13 of 11, 14 of 10) (Avella et al., 2007; DeAngelis & Rhodes, 2016; Madhu et al., 2006). In our anemonefish husbandry system, each breeding pair is maintained in a single tank. As mentioned above, all of these tanks make up one complete rearing system, for which we applied the following parameters: Temperature of 26°C, 34 g/L salinity, and a 14-/10- h light/dark photoperiod. Temperature can be modulated to increase or decrease spawning frequencies and to modulate offspring's developmental rate. However, high temperature should not be constantly applied as it may lead to exhaustion of breeding pairs, which will ultimately reduce reproductive success. Nitrogen compounds ($NH_4$, $NO_2$, and $NO_3^-$) should remain at low levels (0–0.5, 0–0.2, and 0–5 mg/L, respectively) to preserve breeding pair health and the quality of clutches (Callan, 2007; Edwards et al., 2005).

Breeding pairs are fed with a diet that consists of high levels of unsaturated fatty acid which are known to increase fecundity and embryo viability (Callan, 2007). This diet is a homemade mix of squid, mussels, shrimps, pellets (Breeder line OF Crumble, Ocean Nutrition), and nori algae (see Table 1 for a detailed recipe for a 3-kg mix). This mix is conserved frozen and distributed once a day to the breeding pairs. If breeding pairs do not reproduce successfully, it is advised to check all parameters of the husbandry system: Temperature, photoperiod, salinity, pH, and nitrogen compounds. Also, check for any elements that may stress the fish (noise, too many people in the room, etc.).

A terracotta pot in each breeding pair tank is used as a substrate for anemonefish clutches (Figure 1). In 2018, our breeding pairs laid 100–500 eggs every 12–15 days (±1–2). Annual mean was 24 (±4) clutches per breeding pair (Figure 2a,b). We also observed that each year (2018 and 2019) the fish stopped laying eggs for 1 or 2 months (from December to January/February). Eggs hatch 10 days after fertilization in our rearing conditions and hatching occurs 1 h after light extinction.

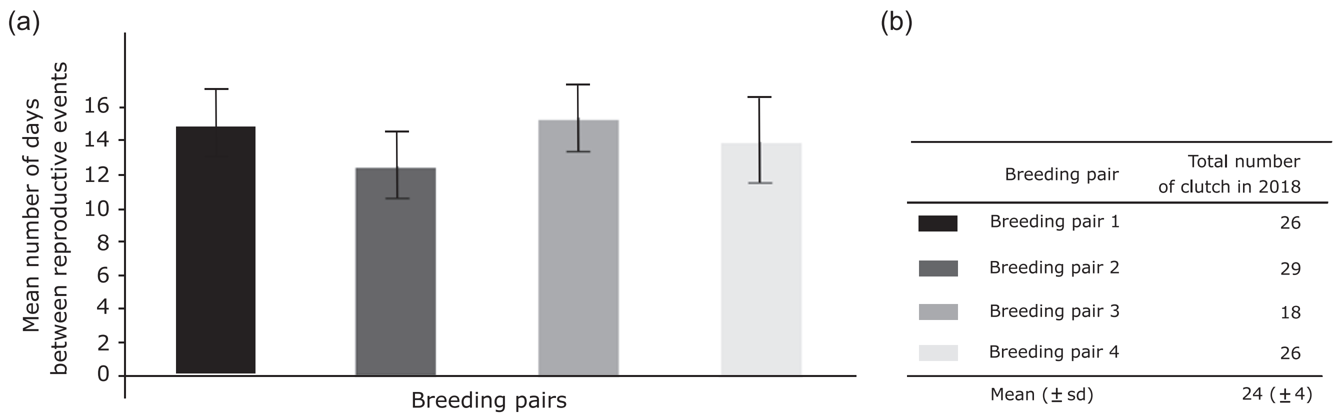
**TABLE 1** Nutritional value and food composition for 3 kg of broodstock homemade diet

| Food | Protein (for 100 g) | Lipid (for 100 g) | Quantity for a 3-kg mix |
|---|---|---|---|
| Mussels | 23.8 g | 4.48 g | 1 kg |
| Shrimps | 21 g | 1.90 g | 1 kg |
| Squid | 32.48 g | 1.4 g | 1 kg |
| Egg yolk | 15.89 g | 32.67 g | 6 yolks (2 per kg of preparation) |
| Pellets | 59 g | 13 g | 300 g (100 g per kg of preparation) |
| Nori algae | 36 g | 1.8 g | 9 g (3 g per kg of preparation) |

## 3 | LIVE PREY AND *NANNOCHLOROPSIS OCCULATA* CULTURING

To ensure a high larval survival rate for *A. ocellaris* it is essential to feed larvae with live prey throughout their development as predatory behavior needs to be stimulated (Calado et al., 2017). Proper management of live prey is, thus, an important prerequisite to rearing anemonefish larvae. Larvae are fed with three types of live prey cultured in the lab: Rotifers (*Brachionus plicatilis*) from hatching (stage 1) to 8 days post-hatching (dph; stage 3/4) (Divya et al., 2011), newly hatched *nauplii* of brine shrimps *Artemia* spp. progressively added from 3 (stage 1/2/3) to 30 dph (juvenile stage) (Dhont et al., 2013), and the green microalgae *N. oculata* from hatching to 8 dph (stage 1 to stage 3/4) (Figure 3). From 30 dph (juvenile stage), *nauplii* are replaced by artificial food (80- and 200-μm pellets, TYC).

Rotifers, which are the conventional live prey for anemonefish laboratory rearing protocols, are cultured in four 12-L buckets filled with the artificial seawater, maintained at a temperature of 25°C and a salinity of 23 g/L (Figure 3a). Rotifers are fed twice per day with 5 ml of RG complete (a mixture of concentrated microalgae—Planktovie). Counts of rotifers and water exchanges are conducted daily to ensure a stable concentration of 250 individuals/ml and sufficient renewal of the population. This means that if the concentration exceeds 250 individuals/ml, daily water removal allows to decrease the concentration to 250 individuals/ml. When concentrations drop below 250 individuals/ml, water exchange is conducted by filtering the rotifers on a 40-μm mesh. These filtered rotifers are then reintroduced into the bucket to increase the concentration. Twice a week, buckets are changed and cleaned with water and 70% Ethanol to avoid any risk of culture contamination. On the day of anemonefish hatching, 600,000 rotifers are harvested and filtered on a 40-μm mesh, rinsed with artificial seawater, and introduced into two beakers filled with 2 L of seawater (23 g/L) and 2 ml of RG complete. Air bubblers are then placed in the beakers to oxygenate the rotifers kept at room temperature. The night of hatching, the 300,000 rotifers in the first beaker are filtered on a 40-μm mesh, rinsed with artificial seawater, and introduced into the larval rearing tank to a concentration of 10 rotifers/ml. The second beaker is then used to feed larvae twice (morning and afternoon) at 1 dph (stage 1). One beaker of 300,000 rotifers in 2 L is then prepared daily for the next day's feeding until 8 dph (stage 3/4, Figure 4). Rotifers are thus distributed twice a day to maintain a constant concentration (10 rotifers/ml) in the larval rearing tank (Avella et al., 2007; Divya et al., 2011). When adding rotifers to the rearing tank, 1 L of water from the beaker is filtered on a 40-μm mesh, rotifers are rinsed with artificial seawater and then introduced into the larval rearing tank.




| Breeding pair | Total number of clutch in 2018 |
|---|---|
| Breeding pair 1 | 26 |
| Breeding pair 2 | 29 |
| Breeding pair 3 | 18 |
| Breeding pair 4 | 26 |
| Mean (± sd) | 24 (± 4) |

**FIGURE 2** *Amphiprion ocellaris* reproduction frequency and a number of clutches. (a) Graph showing the mean number of days between reproductive events of the four breeding pairs in our husbandry system. (b) Total number of clutches obtained for each breeding pair in 2018

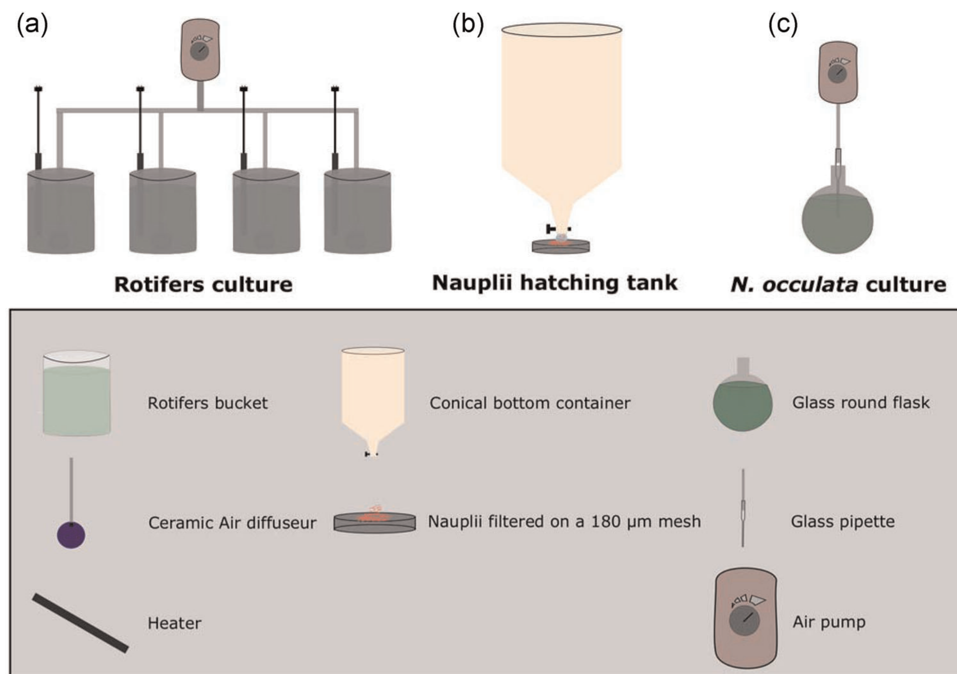



**FIGURE 3** Schematic diagram of the live prey and alga cultures. (a) The rotifer culture; (b) the *nauplii* hatching system; (c) the *Nannochloropsis oculatta* culture

*Nauplii* of the brine shrimp *Artemia* spp. are obtained from dormant cysts (EG artemia, INVE) which are placed for hatching in conical bottom containers filled with natural seawater at 28°C and a salinity of 37 g/L (Figure 3b). Containers are aerated from the center bottom. Each morning, aeration is stopped and newly hatched *nauplii* are placed in the dark so they can concentrate at the bottom of the containers. They are then collected on a 180-µm mesh (Figure 3b), rinsed, and distributed at a concentration of 3 nauplii/ml to anemonefish larvae from 3 to 30 dph (stage 2 to juvenile stage, Figure 4). From 12 dph (stage 4/5/6), artificial food is also given to the larvae starting with 80-µm pellets and progressing to 200-µm pellets (TYC). These pellets are distributed once a day with a ration size of 2% of the body mass of the larvae. The ratio is progressively increased up to 8% of the body mass (Johnston et al., 2002). Finally, big pellets (Ocean nutrition) are given around 30 dph twice a day at a ratio of 6%–8% of the body mass (juvenile stage, Figure 4).

Microalgae are commonly used as live prey in marine fish larval rearing as it is known to enhance survival rates through the provision of nutrients to the larvae, contribute to the preservation of rotifers and *nauplii* nutritional quality and maintain water quality (Tucker, 1998). It has also been proposed that microalgae amplify the visibility of live prey (Naas et al., 1992, 1996). The green microalgae *N. oculata* is thus cultured in our laboratory in 10-L round glass flasks at 18°C, in 7 L of sterile-filtered (0.7 µm) seawater complemented with 1 ml of Conway culture medium (see composition on Table 2) (Figure 3c). Three liters per flask are sampled twice a week during

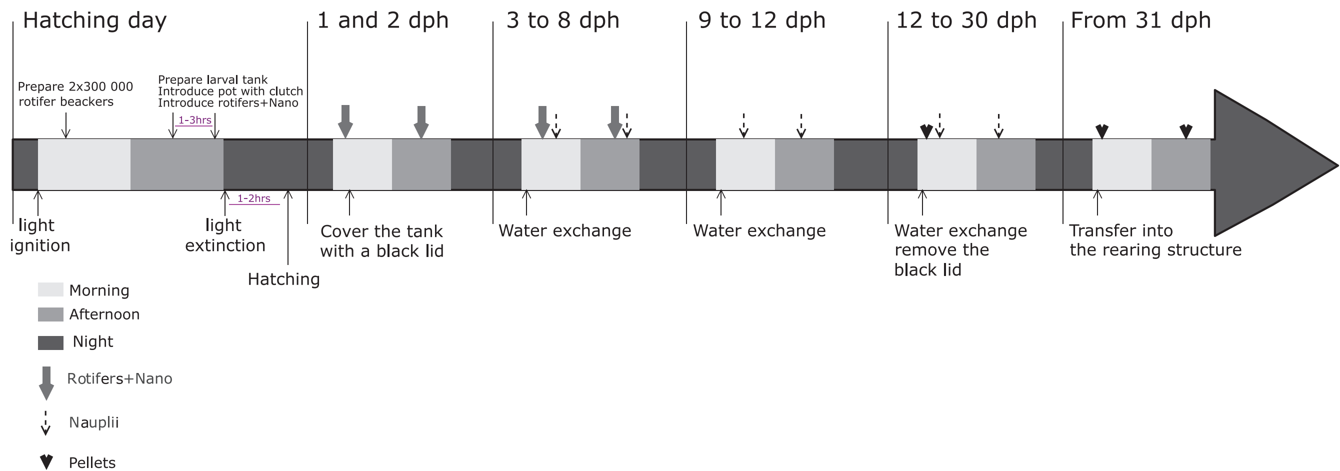



**FIGURE 4** Time series summarizing the main events of rearing anemonefish larvae dph, days post hatching

the exponential growth phase (when the culture is dark green) and concentrated by centrifugation for 30 min at 4°C in 500-ml tubes. Pellets are then diluted in 50 ml of artificial seawater (34 g/L) and stored at 4°C in 50-ml tubes for 7 days. Three liters of culture allow for the preparation of eight 50-ml tubes of concentrated *N. oculata*. A mix of the concentrated *N. oculata* and RG complete (50 + 2 ml RG) is added twice a day to the larval rearing tank from 1 to 8 dph (stage 1 to 3/4) to create live algae green water (Figure 4).

Such cultures do not need to be created in the laboratory as some companies sell a concentrated solution of *N. oculata* which can also be used to create the essential green water environment. However, close attention should be paid to the quality of this solution as it can be altered by temperature variations during transport. Although *N. occulata* is the main species used to rear anemonefishes (and other fish species), it is also possible to use other species such as *Isochrysis sp* and *Chlorella sp* (Divya et al., 2011; Ignatius et al., 2001; Kumar et al., 2010).

**TABLE 2** Chemical composition and recipe of Conway culture medium

| Solution | Chemical elements |
|---|---|
| Metalic Solution (for 100 ml of sterile distilled water) | $ZnCl_2$ (zinc chloride) 2.10 g<br>$CoCl_2$ $6H_2O$ (cobalt chloride) 2 g<br>$6(NH_4)Mo2O_{24}$ $4H_2O$ (ammonium heptamolybdate) 0.9 g<br>$CuSO_4$ $5H_2O$ (copper sulfate) 2 g |
| Principal solution (in 1 L of sterile distilled water) | Metalic solution: 1 ml<br>$Na_2$ EDTA (disodic EDTA) 45 g<br>$NaNO_3$ (sodium nitrate) 100 g<br>$H_3BO_3$ (orthoboric acid) 33.6 g<br>$NaH_2PO_4$ (sodium dihydrogen phosphate) 20 g<br>$MnCl_2$ $4H_2O$ (manganese chloride) 0.4 g<br>$FeCl_3$ $H_2O$ (ferric chloride) 1.3 g |
| Vitamin solution (for 100 ml of sterile distilled water) | Thiamine hydrochloride 200 mg<br>Vitamin B12 10 mg |
| Culture medium (for 1 L of filtered sterile seawater) | 1 ml of principal solution + 0.1 ml of vitamin solution |

## 4 | ANEMONEFISH LARVAE CLASSIC REARING PROTOCOL

Anemonefish larval rearing occurs in the same room as the husbandry but in independent rearing tanks of 54 L (60 × 30 × 30 cm). The rearing tanks are filled with 30 L of seawater from the rearing structure on the day of hatching, immediately before introducing the clutch into the tank (Figure 5a). It is very important to use seawater from the breeding pair tanks to reduce larval mortality. The temperature is maintained with a heater at 26°C, and two bubblers with very low airflow rate (15 L/h) are used to oxygenate the tank and create gentle water movement to avoid live prey sedimentation (Figure 5a). Larval rearing tank walls are surrounded with black plastic bags to reduce light intensity and create a contrasting background that is supposed to increase the visibility of live prey to larvae. A black lid should cover the tank from the morning following hatching until completion of metamorphosis (around 15 dph at 26°C) to reduce distracting light reflections at the surface of the seawater that can attract larvae and cause stress, oxygen depletion, starvation, and, thus, death (Calado et al., 2017; Tucker, 1998).

On the day of hatching, the pot with the clutch is transferred into the rearing tank using a bucket, ensuring the eggs are not taken out of the water (Figure 5a). As hatching occurs 1 h after light extinction, it is advised to transfer the pot between 1 and 3 h before lights are turned off. Once the clutch is placed in the tank, bubblers are placed below the clutch to ensure aeration and movement of eggs until hatching. Newly hatched individuals are kept in the larval rearing tank until 30 dph. When they reach this age, young juveniles are transferred (after an

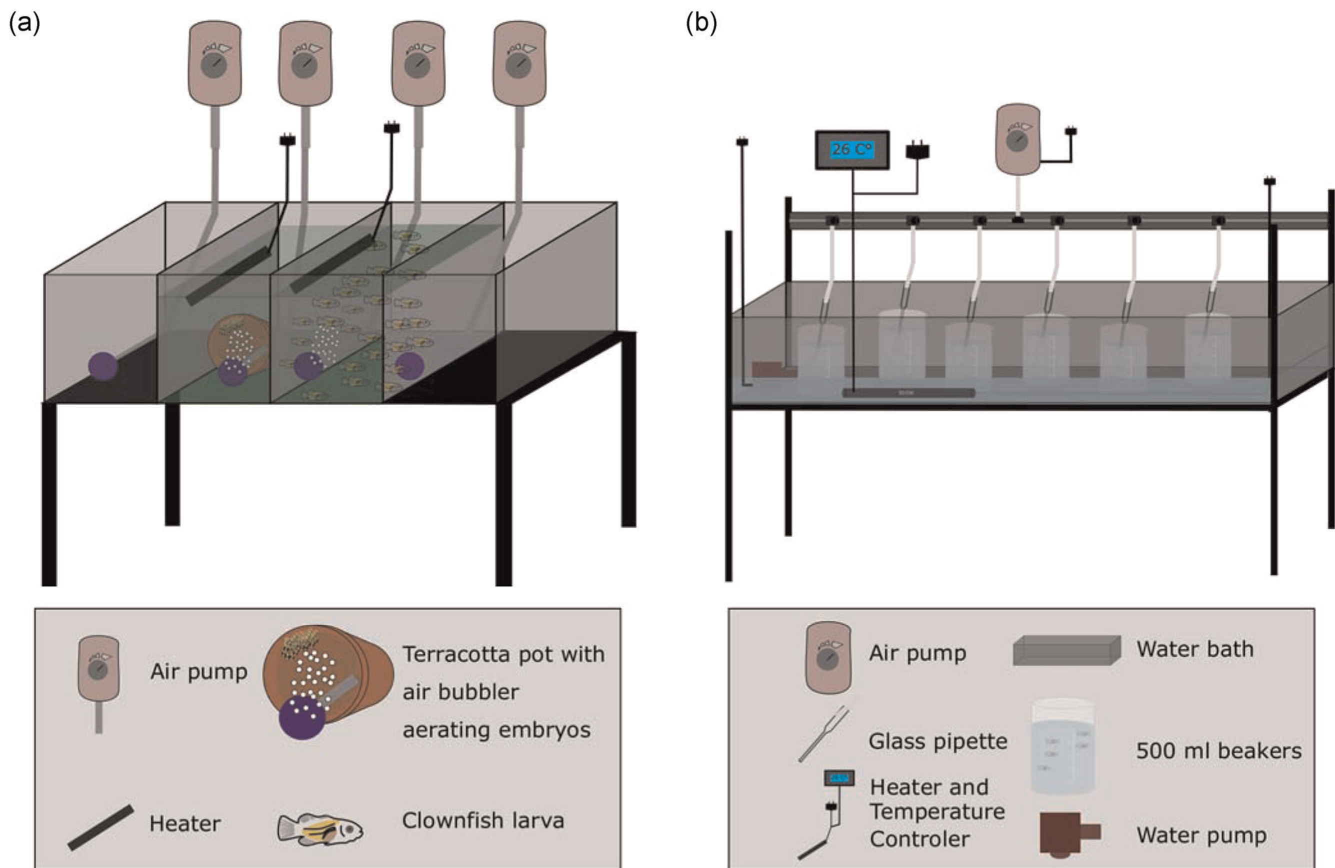



**FIGURE 5** Schematic diagram of the larval tanks and the low-volume rearing set up. (a) Disposal of the terracotta pot in the tank the night of hatching and the larval tank after hatching. (b) Water baths and the experimental beakers, as well as the set-up of the air system

acclimation period of 20 min) into the husbandry, tanks to continue their growth. It is also important to note, that juveniles can become very aggressive and, thus, constant fighting may induce high levels of mortality (up to 90%). To avoid this, we recommend raising the juveniles in small tanks (60 L) with no shelter, to prevent the young fish from settling and establishing a distinct territory.

Daily water exchanges (30% of the total volume) are conducted from 2 dph to carefully remove dead larvae, surplus feed, feces, and waste compounds, thus ensuring a stable quality of seawater. Water exchanges are conducted by slowly adding breeding pair tank seawater (0.6 L/min) through 4- to 5-mm-diameter plastic tubing. It is essential to gently refill the tank to slowly acclimate larvae to the new water conditions. This is vital as they are very sensitive to variations in water parameters. Such a method also limits water movement, which is important as intensive water movement can stress and kill larvae.

## 5 | LOW-VOLUME ANEMONEFISH LARVAE-REARING PROTOCOL

As 30-L tanks are not suitable to conduct pharmacological treatments on anemonefish larvae, we developed a protocol to rear larvae in reduced volumes. For this low-volume rearing protocol, anemonefish larvae are placed in 500-ml glass beakers with permanent aeration supplied by glass pipettes (150 bubbles/min), constant temperature (26°C), and photoperiod similar to those at the husbandry system (14-/10-h light/dark). To maintain a constant temperature, beakers are placed in a heated water bath with circulating water (ensured by a water pump, Aquarium system, MaxiJet 500) (Figure 5b).

Larvae are collected with a glass container at 1 dph (stage 1) and placed in beakers with a maximum density of 5 to 10 larvae/beaker. More than 10 larvae would be fatal to larval survival in such volume. It is important to catch larvae carefully and never siphon them with a tube or collect them with a hand net, as these methods will induce stress and potentially kill them. Glass containers should be transparent allowing for the capture of larvae without touching them, thus limiting their stress. Food supply and water changes are adapted from the classic rearing protocol. Larvae are fed twice a day from 1 (stage 1) to 8 dph (stage 3/4) with rotifers at 10 individuals/ml. A preculture of 50,000 Rotifers is prepared in 1 L of artificial seawater and fed with 1 ml of RG complete. For each food distribution, 500 ml of this preculture is filtered on a 40-µm mesh and diluted in 100 ml of seawater. 1.5 ml of the suspension is then distributed to each beaker. From 3 dph (stage 1/2/3), larvae are also fed with *nauplii* of artemia once per day (15 nauplii/beaker). From 8 dph (stage 3/4), rotifer

addition is stopped, and larvae are fed twice per day with *nauplii* of artemia. *N. occulata* is added, from 1 to 8 dph (stage 1 to stage 3/4), twice per day in beakers (500 µl/beaker) to create green water and enhance survival rates. From 2 dph (stage 1/2), daily water exchanges are done as in the classic rearing protocol to remove dead larvae and ensure a stable quality of seawater (30% of the total volume). Seawater is retrieved from each compartment with a syringe and replaced with seawater from breeding pair tanks. This protocol enabled us to conduct multiple experiments, for example, 30 experiments were launched in 2018 (Salis et al., 2019; Salis, Roux, Soulat, et al., 2018).

## 6 | CONCLUSION

Most teleosts used as model species in Eco-Evo-Devo are freshwater species, meaning there is a clear lack of marine model species in Eco-Evo-Devo, as aquaculture species are not well suited for laboratory-scale rearing. The methods described in this paper, thus, represent a major advancement for using anemonefishes as experimental marine model species and facilitating regular reproduction in a calibrated laboratory-scale system. The rearing system presented here is composed of five larval tanks and 30 tanks to rear breeding pairs and growing juveniles. The entire system can be easily adapted to meet lower needs. The equipment required to set up such a husbandry system is available worldwide, making it possible to establish anemonefishes as a model organism in any laboratory willing to work with these species. For example, our methods have been successfully used to set up a similar anemonefish husbandry system in Taiwan (Marine Research Station, Academia Sinica). The development of the low-volume protocol enabled us to conduct experiments with different pharmacological compounds to investigate *A. ocellaris* metamorphosis and pigmentation changes through larval development, proving its efficiency (Salis et al., 2019). These methods will undoubtedly increase the appeal of using anemonefishes as new marine model organisms in Eco-Evo-Devo. This is demonstrated by recent studies describing the larval development of *A. ocellaris* and *A. perideraion* as well as the evolution of pigmentation patterns in anemonefish (Roux et al., 2019; Salis, Roux, Lecchini, et al., 2018; Salis, Roux, Soulat, et al., 2018). These methods can also be used to expose anemonefish larvae to different environmental perturbations, thus investigating how they will respond to future global change and anthropogenic pressures. Successful larval development is essential to the maintenance of coral reef fish populations and anemonefish will provide an excellent Eco-Evo-Devo experimental model for coral reef fishes.

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### CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

### AUTHOR CONTRIBUTIONS

Natacha Roux and Valentin Logeux wrote the manuscript and developed the low-volume rearing protocol. Nancy Trouillard, Pauline Salis, and Natacha Roux set up the rotifers culture in the Observatoire océanologique of Banyuls-sur-mer. Nancy Trouillard also set up and maintained the *N. occulatta* culture. Natacha Roux, Valentin Logeux, Kévin Magré, Rémi Pillot, Pauline Salis, and Pascal Romans participated in the construction of the husbandry. Natacha Roux and Pauline Salis participated in the maintenance of the husbandry and the rotifer culture at the beginning of their establishment. Valentin Logeux is in charge of husbandry maintenance and larval rearing, Rémi Pillot and Nancy Trouillard also assisted with larval rearing. Natacha Roux, Pauline Salis, and Laurence Besseau are in charge of the low-volume experiments. Pascal Romans was in charge of the acquisition of *A. ocellaris* breeding pairs. The figures were created by Natacha Roux. Vincent Laudet and David Lecchini established anemonefish as an experimental model. All the coauthors read, corrected, and approved this manuscript.

### ETHICS STATEMENT

We have approval for these experiments from the C2EA—36 Ethics Committee for Animal Experiment Languedoc-Roussillon (CEEA-LR), number A6601601. We have an approval number of premises for animal testing issued by the Regional Directorate of Food, Agriculture, and Forestry of Occitania and the Departmental Directorate of Protection of Populations of the Pyrenees Orientales. The animals reared in our husbandry are from breeding stock.

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