24 Anemonefishes

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24.1 HISTORY OF THE MODEL

> I noticed a very pretty little fish which hovered in the water close by, and nearly over the anemone. This fish was six inches long, the head bright orange, and the body vertically banded with broad rings of opaque white and orange alternately, three bands of each. As the fish remained stationary, and did not appear to be alarmed at my movements, I made several attempts to catch it; but it always eluded my efforts, not darting away, however, as might be expected, but always returning presently to the same spot. . . . I visited from time to time the place where the anemone was fixed, and each time, in spite of all my disturbance of it, I found the little fish there also. This singular persistence of the fish to the same spot, and to the close vicinity of

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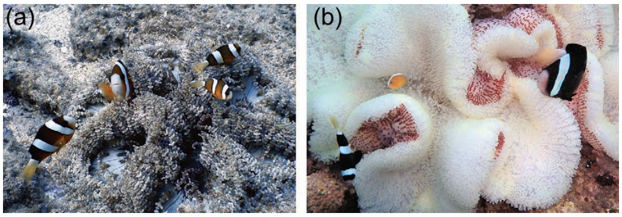

FIGURE 24.1 Colony of *A. clarkii* (a) and cohabitation of *A. clarkii* and *A. sandaracinos* (b) in Okinawa, Japan. ([a] Photo courtesy of Manon Mercader; [b] photo courtesy of Kina Hayashi.)

> the great anemone, aroused in me strong suspicions of the existence of some connection between them.
>
> **(Collingwood 1868)**

This is the first written description of an anemonefish* (Figure 24.1) and its peculiar lifestyle, observed by English naturalist Cuthbert Collingwood in 1866 at Fiery Cross Reef off the coast of Borneo. The remarkable symbiosis between anemonefishes and giant sea anemones has since then received a lot of attention, becoming one of the main examples of mutualistic interactions (Apprill 2020). It is actually the keen interest for this interaction that first drove scientists to study these fish (Mariscal 1970; Lubbock and Smith 1980; Fautin 1991), but, as scuba diving became popular, rending shallow environments easily accessible, multiple aspects of their biology and ecology soon started to be investigated (Mariscal 1970; Allen 1974; Moyer 1980; Ochi 1985; Murata et al. 1986). Indeed, anemonefishes are unthought-of models for marine ecologists as, unlike many marine fishes, they can be easily located at a given site as well as followed through time. Besides, they are also relatively easy to capture and, being one of the most iconic tropical reef fish species, they quickly became a must-have for aquarium hobbyists. They were one of the first captive-bred marine fish back in the 1970s, and now, many species as well as a variety of fancy mutants can easily be found in pet shops. This combination of efficient rearing and convenient sampling possibilities makes anemonefishes excellent model organisms not only for marine ecologists but also for a multitude of biological fields (reviewed in Roux et al. 2020). Until now, studies on behavior (Buston 2003a; Rueger et al. 2018), physiology (Park et al. 2011; Miura et al. 2013), development (Salis et al. 2018b; Roux et al. 2019b), evolution (Litsios et al. 2012a; Rolland et al. 2018) and population dynamics (Nanninga et al. 2015; Salles et al. 2015), just to mention a few, have been conducted using anemonefishes.

24.2 GEOGRAPHICAL LOCATION AND PHYLOGENY

Anemonefishes form a clade of at least 30 species in genera *Premnas* and *Amphiprion*, including two species that are natural hybrids (*A. leukokranos* [*A. sandaracinos* X *A. chrysopterus*] and *A. thiellei* [*A. sandaracinos* X *A. ocellaris*]) within the Pomacentridae family (Frédérich and Parmentier 2016). All are living as symbionts with ten sea anemone species that belong to three distantly related families (*Thalassianthidae, Actinidae, Stichodactilidae*) (Allen 1974; Fautin and Allen 1997; Ollerton et al. 2007; Allen et al. 2008, 2010). This mutualistic relationship is the driving force of their diversification through adaptive radiation (Litsios et al. 2012b). However, diversification of giant sea anemones occurred before the establishment of this symbiotic relationship. Since their taxonomy is still unclear, the specificity between anemonefishes and their hosts will likely be revisited (Titus et al. 2019; Nguyen et al. 2020).

Historically, anemonefishes were categorized into six morphology-based groups; genus *Premnas* formed a group on its own, and *Amphiprion* was divided into four subgenera: *Actinicola, Paramphiprion, Phalerebus* and *Amphiprion* (the last one sub-divided into two species complex: *ephippium*-complex and *clarkii*-complex) (Allen 1974; Allen et al. 2008, 2010). It was also believed that the ancestral anemonefish was able to live in association with multiple sea anemone species (i.e. generalist) that later radiated into various more specialized species (Elliott et al. 1999). This process is commonly used to explain the evolution of symbiotic organisms (Futuyma and Moreno 1988). *A. clarkii* was then believed to be at the base of the anemonefish phylogenetic tree, as it is the most widespread and generalist species of the tribe. It is also less dependent on its host sea anemone due to its good swimming performance and its morphology, which resembles that of other free-living pomacentrids. However, the latest molecular phylogenetic studies do not support those hypotheses based on morphological traits. They support the monophyletic origin of anemonefish species, but the topologies found are inconsistent with the grouping into the six complexes mentioned previously. They also place *A. percula* and *A. ocellaris*, both specialists and poor swimmers, at the basal node of the tree (Santini and Polacco 2006; Litsios et al. 2012a, 2014b) (Figure 24.2a).

All 30 species of anemonefish inhabit coral reef environments in the warm, tropical waters of the Indo-Pacific Ocean, from Australia to the Ryukyu archipelago and from Thailand to the Marshall Islands (Figure 24.2 B) (Allen 1974; Fautin and Allen 1992, 1997; Allen et al. 2008, 2010). Distribution varies greatly from one species to another, with some being widespread (e.g. *A. clarkii, P. biaculeatus*) (Figure 24.2c), while others have a restricted regional distribution (e.g. *A. bicinctus, A. percula*) (Figure 24.2d) or are even confined to a few islands (e.g. *A. chagosensis, A. fuscocaudatus*) (Figure 24.2e). The highest diversity is found in the Coral Triangle (Fautin 1988; Elliott & Mariscal 2001; Camp et al. 2016), which is probably their center of origin (Santini and Polacco 2006; Litsios et al. 2014b). In the Madang region (Papua New Guinea), nine species of anemonefish can be found in sympatry. Such coexistence is explained by niche differentiation, species coexisting through resource partitioning by using different host anemone species and/or habitat (e.g. depth, localization in the reef). They can even

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* The term anemonefishes, rather than clownfishes, is used in this chapter to refer to *Amphiprion* and *Premnas* even though other fishes (pomacentrid and also non-pomacentrid; Randall & Fautin 2002) can eventually live in sea anemones. This choice was made to avoid confusion due to the variety of common names employed for the different species of this clade.

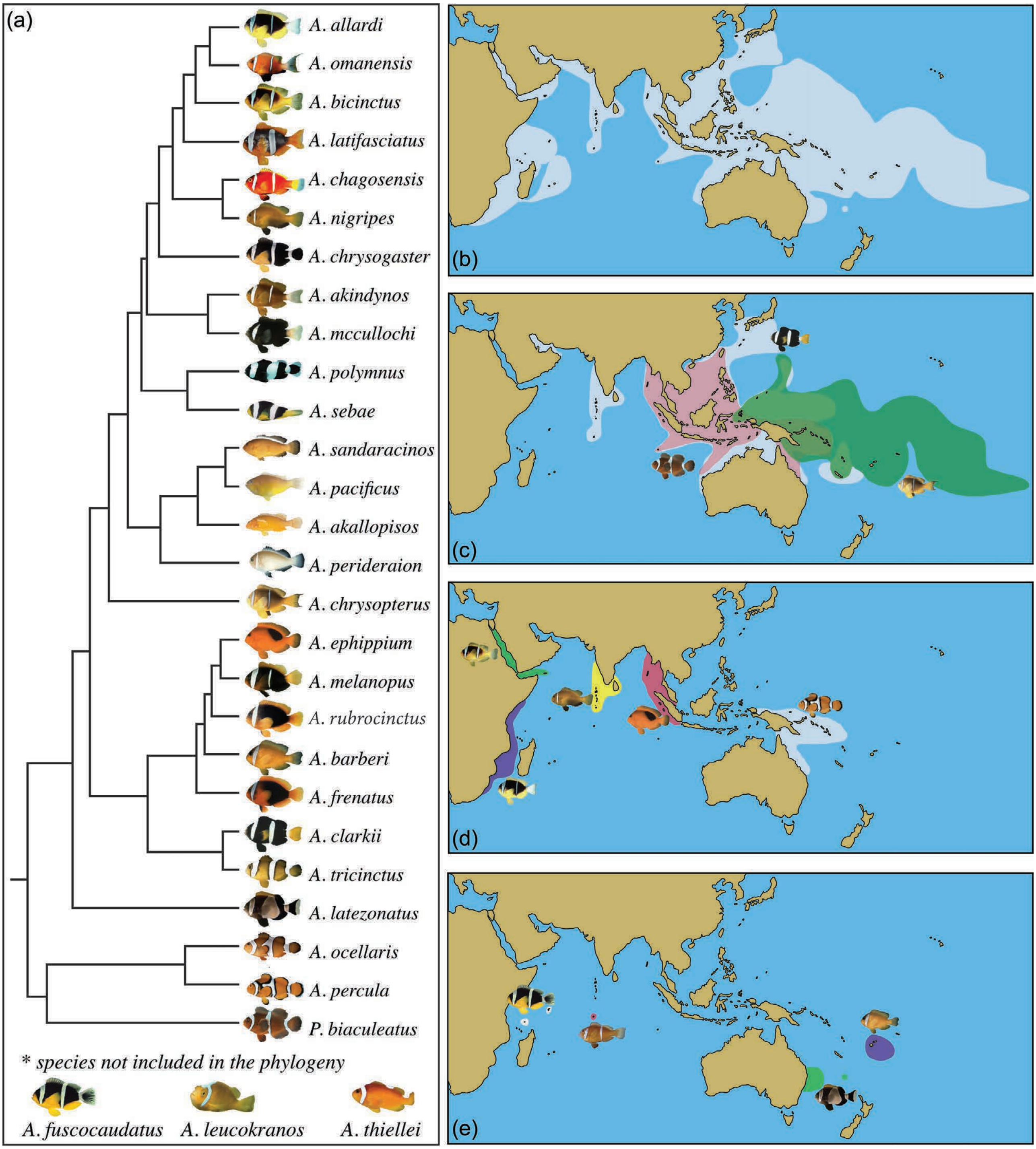

FIGURE 24.2 Phylogenetic relationship and geographic distribution of anemonefishes. Phylogenetic tree of 27 anemonefish species. Three species could not be included in the tree because they are either rare (*A. fuscocaudatus*) or hybrid species (*A. leucokranos* and *A. thiellei*) (a) Anemonefishes are distributed across the Indo-Pacific Ocean (b), with some species being widespread, such as *A. chryosopterus, A. clarkii* and *P. biaculeatus* (c); regional, such as *A. allardi, A. bicinctus, A. ephippium, A. nigripes* and *A. percula* (d); or restricted to specific areas, such as *A. barberi, A. chagosensis, A. fuscocaudatus* and *A. latezonatus* (e). (Adapted from the published work of Litsios et al. 2014b; Rolland et al. 2018.)

coexist in the same anemone (Figure 24.1b) by partitioning space in it (Elliott and Mariscal 2001; Camp et al. 2016; Hayashi et al. 2018). Anemonefishes can also be found in the Red Sea, the southwest coasts of Africa, the Maldives, French Polynesia and as far north as the southeast coast of Japan, where the warm Kuroshio current carrying tropical waters provide them adequate conditions (Moyer 1976; Fautin and Allen 1992; Fautin and Allen 1997). According to their evolutionary history, anemonefishes first spread from the Coral Triangle and then colonized the Indian and central Pacific Oceans, where they diversified around four million years ago (Mya), leading to their present distribution and diversity (Litsios et al. 2014b). In accordance with this model, farther from the coral triangle, species richness declines (Camp et al. 2016). While six species can still be found in sympatry in Okinawa (Japan) (Hayashi et al. 2018) or Lizard Island (Great Barrier Reef), only one is living in the Red Sea or French Polynesia (Allen 1974; Fautin 1988; Elliott and Mariscal 2001). Anemonefishes are not found in some Pacific islands such as the Hawaiian Islands, Johnston Atoll and the Marquesas (Randall 1955), nor on the coast of Central and South America or the Atlantic. This pattern of distribution is common to many Indo-Pacific species, which are unable to disperse past the East Pacific Barrier (Briggs 1961; Robertson et al. 2004). Since anemonefishes are obligate symbionts, their distribution is strictly dependent on their Actinian host's distribution and specific habitat requirements. Due to their endosymbiotic zooxanthellae host, sea anemones are restricted to the photic zone (≤200 m), and therefore anemonefishes are mainly found in clear shallow waters, usually no deeper than 50 m.

24.3 LIFE CYCLE

Anemonefishes exhibit the classical bi-partite life cycle of most reef fish, which is composed of a pelagic dispersive larval phase followed by a demersal juvenile and adult phase (Leis 1991) (Figure 24.3). However, their peculiar lifestyle distinguishes them from other species.

Anemonefishes live in socially well-structured colonies composed of a dominant breeding pair and several immature individuals (Figure 24.1a). A sized-based dominance hierarchy structures each colony; the largest fish is a dominant female, which defends the colony, and the second largest is a sub-dominant male taking care of the demersal eggs (Olivotto and Geffroy 2017). This monogamous pair is surrounded by smaller, sexually immature individuals, ranked by size, the smallest (youngest recruit) being at the bottom of the hierarchy (Fautin and Allen 1992; Buston 2003a; Iwata et al. 2012; Casas et al. 2016; Olivotto and Geffroy 2017). Anemonefishes have been described as protandrous sequential hermaphrodites, and the sex change from functional male to female is size dependent and/or socially mediated (Fricke and Fricke 1977). When the female disappears from the group, the male changes sex, and the third-ranked fish inherits the male breeding position and territory, thus forming a new monogamous pair (Buston 2004b; Mitchell 2005). Therefore, the size hierarchy represents a queue to attain dominant status and reproduction, individuals only ascending in rank when a higher-ranked individual disappears (Rueger et al. 2018).

Reproduction occurs all year around (except in extreme parts of their distribution range, where reproduction stops during winter), every two to three weeks, usually a week before or after a full moon (Seymour et al. 2018). The breeding couple adopts a specific behavior, which varies among species but generally includes male and female swimming close to each other and touching bellies. This "parade" is initiated by the female, which subsequently lays between 100 and 1,000 eggs, depending on species and conditions, in a roughly circular patch that are immediately fertilized by the male (Allen 1974; Buston and Elith 2011). Eggs are attached to a rock in the direct vicinity of the host sea anemone. This makes anemonefish benthic spawners, unlike most coral reef fish that spawn in the open ocean.

Embryonic development lasts between seven and ten days, during which mainly the male takes care of the eggs by fanning and mouthing them, removing dead ones (which are eaten) and keeping the nest clean (Allen 1974). Hatching occurs just after dusk, and larvae disperse in the open ocean for up to 15 days. The embryonic phase of anemonefish development is rather long compared to other fish species even when compared to other Pomacentridae (e.g. one day for the night sergeant *Abudefduf taurus*, three days for the threespot dascyllus *D. trimaculatus*) (Kavanagh and Alford 2003). Therefore, hatching larvae already have the ability to swim, feed and catch prey merely hours after hatching (Putra et al. 2012). This makes anemonefish larval development one of the shortest known for coral reef fishes (for instance, most pomacentrids have a pelagic larval duration [PLD] that lasts approximately 25 days) (Victor and Wellington 2000; Berumen et al. 2010).

After this dispersive pelagic phase, larvae metamorphose into juvenile individuals. Metamorphosis is a crucial developmental step mediated by thyroid hormones, during which morphological, physiological, behavioral and ecological changes lead to the loss of larval attributes (Laudet 2011). At this time, juveniles look like small adults and leave the open ocean to enter the reef, a process known as recruitment (Figure 24.3). More details on embryonic and larval development as well as on metamorphosis are provided in Section 24.4. Once recruited to the reef, juveniles actively search for an adequate sea anemone using environmental cues and their sensory abilities (Leis et al. 2011; Paris et al. 2013; Barth et al. 2015) to settle and establish the fascinating symbiosis that is so typical of anemonefishes.

The long-term association between anemonefishes and their sea anemones is considered a mutualistic relationship, as the sea anemone provides protection to the anemonefishes, which in turn provide nitrogen and carbon to their host and its endosymbiotic zooxanthellae (playing an important role in their nutrition) (Cleveland et al. 2011), provide protection against predators (mainly butterflyfishes) (Fautin 1991) and reduce hypoxia through aeration-like behavior (Herbert et al. 2017).

This association has always intrigued scientists for two main reasons. First, there is a complex species specificity of this mutualistic relationship, probably related to the toxicity levels of the hosts (Litsios et al. 2012b; Nedosyko et al. 2014; Marcionetti et al. 2019). A few anemonefish species live only in one sea anemone species, such as *A. sebae* and *P. biaculatus* (i.e. specialists). On the contrary, other species may have two or even ten possible hosts such as *A. ocellaris,*

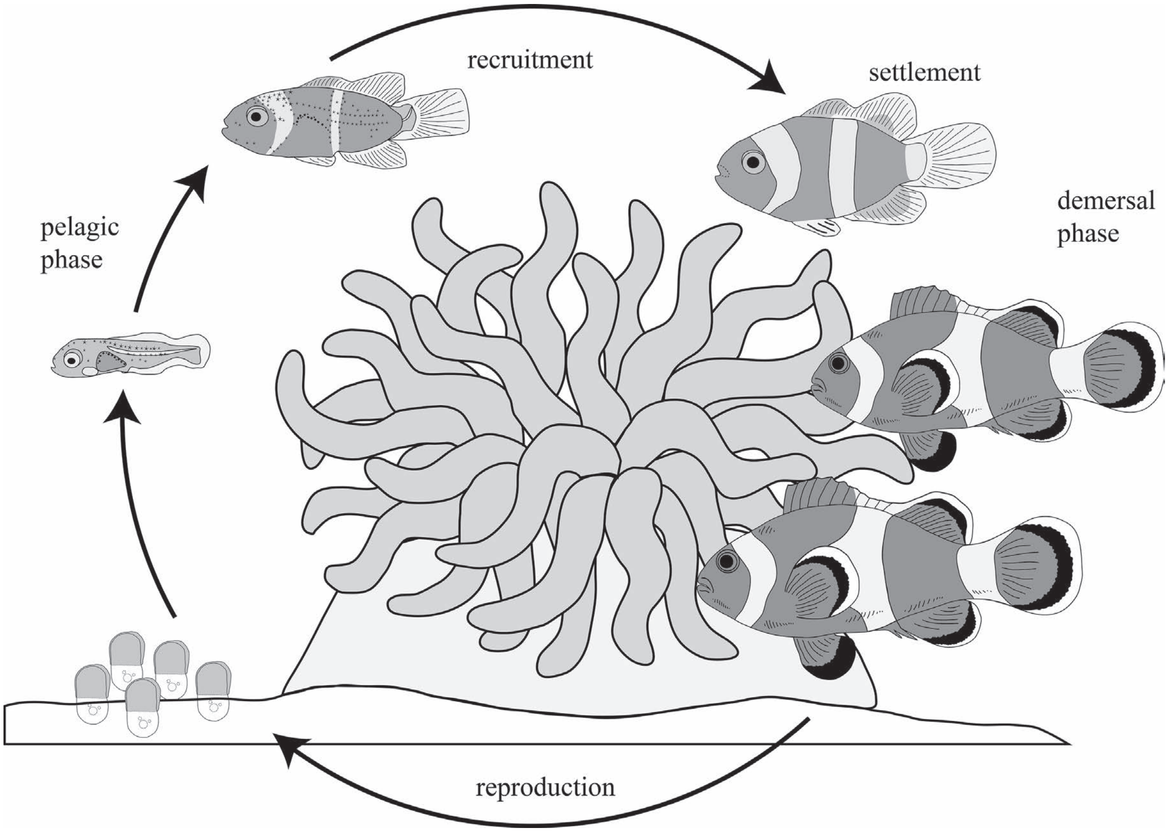

FIGURE 24.3 Anemonefish life cycle. Eggs are laid on the substrate close to the host sea anemone. After hatching, the pelagic larvae disperse in the open ocean. Recruitment to the reef coincides with metamorphosis from larvae to juveniles, which then settle into a sea anemone.

A. bicinctus, A clarkii and *A. perideraion* (i.e. generalists) (Fautin and Allen 1997) (Table 24.1).

Second, anemonefishes are able to live unharmed inside the tentacles of their host, which are known to discharge stinging cells called nematocysts (Mebs 2009). Two main hypotheses have been formulated to explain this ability. The first one suggests that anemonefishes coat themselves with sea anemone mucus, which is therefore used as a chemical camouflage (Fautin 1991; Scott 2008). This is achieved during an acclimation process that corresponds to a series of behaviors during which anemonefishes carefully enter their hosts (Schlichter 1968). First, they kiss the tentacles, then touch them with their pectoral fins and finally scrub their entire body against the tentacles. This behavior has been observed in several species, but not all, and it also seems different depending on the sea anemone species. Surprisingly, *A. clarkii* needs to acclimate when entering in *Entacmea quadricolor* but not when entering the more toxic *Stichodactyla haddoni* (Lubbock 1981; Elliott and Mariscal 1997; Mebs 2009). The second hypothesis suggests that anemonefishes are protected from sea anemone stinging by their own mucus that either prevents nematocyst discharge or protects the fish from the consequence of the discharge. Indeed, it has been shown that *A. ocellaris* lacks *N*-acetylneuraminic acid in its mucus, which is normally detected by sea anemone tentacles to discharge stinging cells (Abdullah and Saad 2015). All these studies suggest that the mucus of both partners is the key to understanding how anemonefishes are able to live in sea anemones without being harmed. Moreover, it has recently been demonstrated that changes in the microbial composition are occurring in both partners during initiation of the symbiosis, suggesting a potential role of bacterial communities in the establishment of this relationship (Pratte et al. 2018; Roux et al. 2019a).

After settlement, anemonefishes integrate into the colony hierarchy, queuing for breeding positions. Why and how anemonefishes engage in such a social system is starting to be understood thanks to extensive work on *A. percula* colonies and may have a great contribution to the understanding of complex societies. Buston and collaborators have shown that members of a colony are not composed of close relatives (2007) and that non-breeders don't provide alloparental care, their presence having neither a positive or negative effect on the dominant pair's breeding success (Buston 2004a). Nonbreeders can adjust their size and growth rate in order to maintain a clear size difference with respect to individuals of higher social rank so that conflicts are limited, thereby reducing the risk of eviction and the potential cost to the breeding dominant pair (Buston 2003a). Consequently, there seem to be no direct benefits of living in such social groups. However, withholding reproduction by staying small and not contesting to remain part of the colony might represent a better option than either leaving the host anemone to breed elsewhere (because of predation risk) or contesting for breeding

TABLE 24.1

Summary of host anemone specificity among all 30 members of the clade (*A. – Amphiprion, P. – Premnas*).

	C. adh	***E. qua***	***H. aur***	***H. cri***	***H. mag***	***H. mal***	***M. dor***	***S. gig***	***S. had***	***S. mer***
A. akallopisos					■					■
A. akindynos		■	■	■	■				■	■
A. allardi		■	■							■
A. barberi		■		■						
A. bicinctus		■	■	■	■			■		■
A. chagosensis		▒								
A. chrysogaster			■		■				■	■
A. chrysopterus		■	■	■	■		■		■	■
A. clarkii	■	■	■	■	■	■	■	■	■	■
A. ephippium		■		■						
A. frenatus		■								
A. fuscocaudatus										■
A. latezonatus				■						
A. latifasciatus										■
A. leucokranos				■	■					■
A. mccullochi		■								
A. melanopus		■		■	■					
A. nigripes					■					
A. ocellaris					■			■		■
A. omanensis		■		■					■	
A. pacificus					■					
A. percula				■	■			■		
A. perideraion				■	■		■	■		
A. polymnus				■			■		■	
A. rubrocinctus		■						■		
A. sandaracinos				■						■
A. sebae									■	
A. thiellei				▒						▒
A. tricinctus		■	■	■						■
P. biaculeatus		■								

* *C. adh – Cryptodendrum adhaesivum, E. qua – Entacmaea quadricolor, H. aur – Heteractis aurora, H. cri – Heteractis crispa, H. mag - Heteractis magnifica, H. mal – Heteractis malu, M. dor – Macrodactyla doreensis, S. gig – Stichodactyla gigantea, S. had – Stichodactyla haddoni, S. mer – Stichodactyla mertensii*

(because of the risk of being evicted or even killed; Buston 2003b; Rueger et al. 2018). Moreover, long-term benefits can come from staying in the colony, as subordinates will inherit the territory in which they reside after the death of breeding individuals (Buston 2004b).

Once they are finally able to reach the highest hierarchical rank, anemonefishes have to undergo a protandrous sex change (from functional male to functional female). Hermaphroditism is widely found in at least 27 teleost families, including Pomacentridae. Indeed, among vertebrates, teleost fish exhibit the greatest diversity in sex determination in relation to a remarkable plasticity of gonadal development and sexual expression (Munday et al. 2006; Liu et al. 2017; Ortega-Recalde et al. 2020).

However, even though the social hierarchy of anemonefishes has been well described for several species, the internal mechanisms at play during protandrous sex change are still poorly understood. Nonetheless, one of the main advantages of anemonefishes as model organisms is that sex change can be experimentally induced, both in field and laboratory conditions, by simply removing the dominant female. It is thus possible to study the molecular and physiological mechanisms governing sex change by following the dominant male during its transition into a functional female.

Histological analysis of gonads revealed that juveniles develop bisexual gonads, otherwise known as ovotestis, possessing both male and female tissues which are topographically distinct but not separated (Kobayashi et al. 2013; Todd et al. 2016; Gemmell et al. 2019). Once sexual maturity is reached, the ovotestis of the reproducing male exhibits a functional male territory, where spermatogenesis occurs, and an immature female territory (Kobayashi et al. 2010). During protandrous sex change, oogenesis occurs in the developing female area of the ovotestis, while the male territory progressively disappears (Casas et al. 2016). This histological scenario of gonadal protandrous transition is the same for all species of anemonefish studied so far (Godwin 1994; Kobayashi et al. 2013; Casas et al. 2016). Studies have reported that cellular changes within the ovotestis are subjected to endocrine control during sex change (Kobayashi et al. 2010; Miura et al. 2013). Like in other sequential hermaphroditic fish, the gonadal sex change is accompanied by major shifts in plasma levels of sex steroid hormones, mainly characterized by a decrease of 11-ketotestosterone levels and a subsequent 17β-estradiol increase (Godwin and Thomas 1993; Miura et al. 2013). Even though observed experimentally, the upstream mechanisms controlling the shift in sex steroid secretion still remain poorly understood. It has been suggested that the crosstalk between the hypotholamo-pituitary-gonadal (HPG) and hypothalamo-pituitary-interenal (HPI) axes plays a central role in the neuroendocrine regulation of protandrous sex change in anemonefishes (Godwin et al. 1996; Lamm et al. 2015). The association between stress and hermaphroditism was first described in *A. melanopus*, in which a peak of serum cortisol levels were observed during later sex change stages (Godwin and Thomas 1993; Goikoetxea et al. 2017; Geffroy and Douhard 2019).

Natural mortality of adult anemonefishes is very low compared to other coral reef fishes, which is most probably due to them being protected from predators by living within their host anemone. Mortality rate is not affected by environmental (e.g. reef, depth, anemone diameter) or demographic (e.g. number of individuals, density and standard length) parameters (Buston 2003b). However, it differs according to the hierarchical rank occupied by the fish. Since low-ranked individuals can be evicted from the anemone and thus undergo greater predatory pressure, juveniles suffer higher mortality than dominant individuals (Buston 2003b; Salles et al. 2015). Standard evolutionary theories of aging (i.e. mutation accumulation, antagonistic pleiotropy and disposable soma theory) predict that low extrinsic mortality leads to the evolution of slow senescence and an extended lifespan (Medawar 1952; Williams 1957; Kirkwood 1977). Anemonefishes are a great example confirming these theories, with some species having been observed to live over 20 years (Sahm et al. 2019), while predictions estimate a lifespan of up to 30 years (Buston and García 2007). Such longevity is exceptional for small fishes and at least twice the estimated longevity for other pomacentrids (Buston and García 2007; Sahm et al. 2019).

24.4 DEVELOPMENT

Anemonefish eggs are capsule shaped, and their size varies depending on the species, with a length from 1.3–1.5 mm (*A. ephippium*) to 2.4–2.6 mm (*A. nigripes*) and a width from 0.53–0.72 mm (*A. ephippium*) to 1.0–1.2 mm (*A. percula*) (Dhaneesh et al. 2009; Anil et al. 2012; Krishna 2018). The developing embryo is separated from a large amount of yolk (i.e. polylecithal, telolecithal egg), which is colored yellow to orange or even red (due to the presence of carotenoids), similar to the parent coloration. The side of the egg that is attached to the substrate (via a glutinous substance and/or threads) has consistently been recognized as the animal pole. Fertilization activates the egg and is characterized by cytoplasmic movements, which result in the formation of a dome-shaped blastodisc (Yasir and Qin 2007; Thomas et al. 2015; Krishna 2018). The chorion is transparent and leaves a narrow perivitelline space. Embryonic development usually lasts between six and eight days, depending on species and temperature. Major developmental changes will be described for all species, as they are very similar to each other, only differing in the exact timing. The following species and literature were compared for this: *A. akallopisos* (Dhaneesh et al. 2012), *A. bicinctus* (Shabana and Helal 2006), *A. ephippium* (Krishna 2018), *A. frenatus* (Ghosh et al. 2009), *A. melanopus* (Green 2004), *A. nigripes* (Anil et al. 2012), *A. ocellaris* (Liew et al. 2006, Yasir and Qin 2007, Madhu et al. 2012, Salis et al.), *A. percula* (Dhaneesh et al. 2009), *A. polymnus* (Rattanayuvakorn et al. 2005) and *A. sebae* (Thomas et al. 2015; Gunasekaran et al. 2017). To avoid disruption, these studies will not be cited again in the following descriptions.

24.4.1 EMBRYONIC STAGE 1: EARLY CLEAVAGES (FIGURE 24.4A)

This stage comprises four synchronous division cycles that lead from a zygote to a 16-cell stage. All blastomeres of a given cell stage are of equal size. Cleavages are meroblastic (partial cleavage) and discoidal (cleavage furrows do not penetrate the yolk). The yolk exhibits prominent fat/oil globules throughout these cleavages.

24.4.2 EMBRYONIC STAGE 2: LATE CLEAVAGES (FIGURE 24.4B)

This stage comprises the division of the 16-cell stage until the start of gastrulation. All blastomeres are of equal size, partially overlapping each other as they arrange themselves into several layers (sphere shape) before they start to spread. The fat/oil globules decrease in number and size and are typically located toward the vegetal pole.

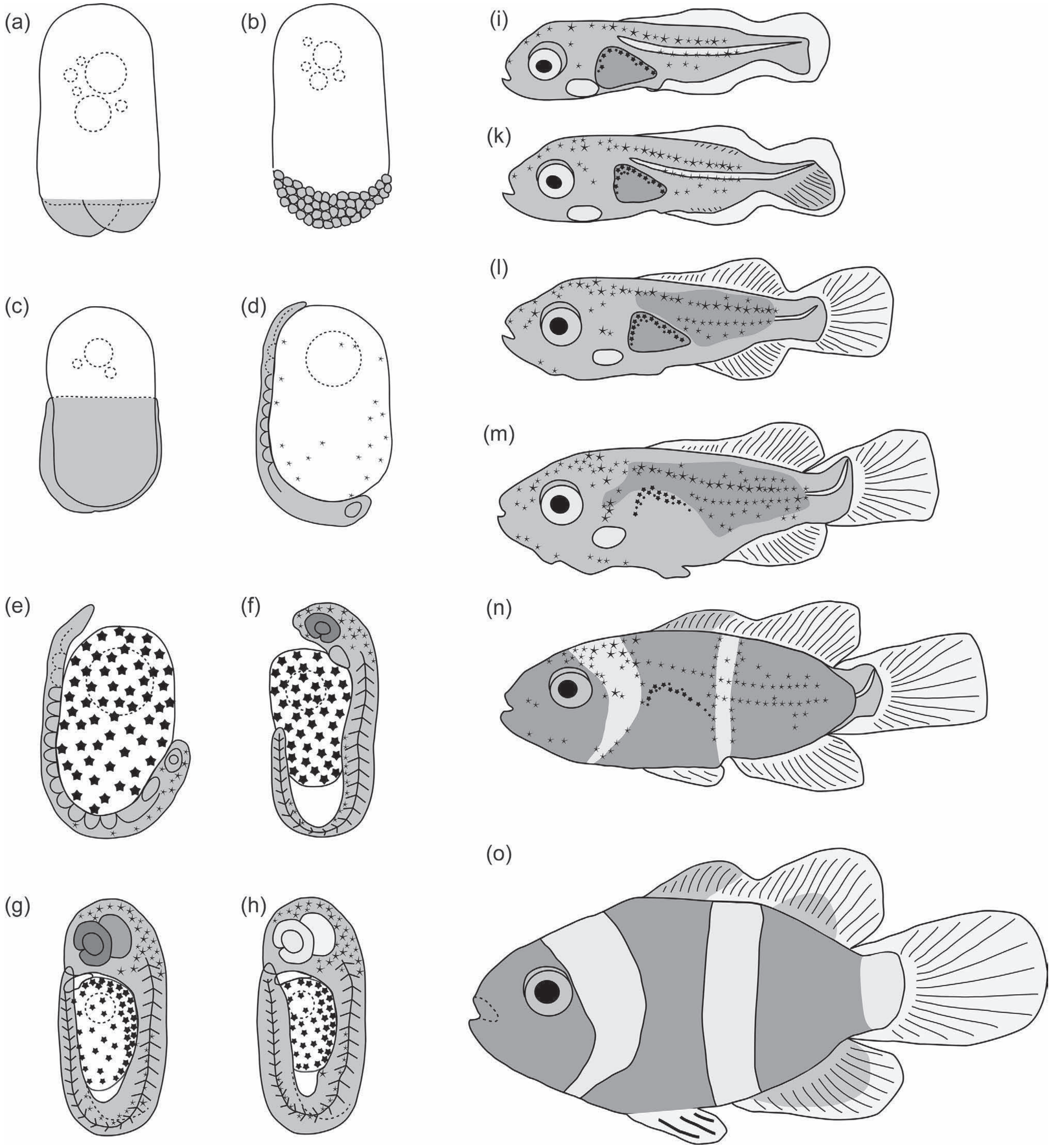

FIGURE 24.4 Embryonic (a–h) and larval (i–o) development of anemonefishes. The schematic drawings of embryonic stages are representative for all anemonefish species and do not refer to a single species, whereas *A. ocellaris* was used as representative for larval schematics (according to Roux et al. 2019b).

24.4.3 EMBRYONIC STAGE 3: GASTRULATION (FIGURE 24.4C)

This stage comprises gastrulation, the formation of the three germ layers: ectoderm, mesoderm and endoderm. During the first step, epiboly, blastomeres flatten, move and extend toward the vegetal pole, covering the underlying yolk. Terms like 50% or 75% epiboly describe how much yolk has been covered by the blastoderm (i.e. the connective sheet of blastomeres). Formation of the embryonic shield, the future embryo, is achieved by a local thickening of blastomeres during 30–75% epiboly.

24.4.4 Embryonic Stage 4: Cephalization and Somite Development (Figure 24.4d)

The head, including optic buds (located at the animal pole), as well as neural ectoderm, is formed. The tail bud begins to develop later on. Overall, this stage marks the beginning of organogenesis and metamerization. The first appearance of paired somites occurs before 100% epiboly is reached (around 60–80% epiboly). Stellate melanophores begin to cover the yolk.

24.4.5 Embryonic Stage 5: Turn-Over (Figure 24.4e)

The entire body of the embryo is covered with few melanophores, particularly abundant in the head region. The head is clearly distinguishable, and the brain has differentiated into three parts: the prosencephalon, mesencephalon and rhombencephalon. Primitive optic buds/vesicles have formed, with subsequent induction of eye formation (eye cup, lens and cornea). Somitogenesis (trunk segmentation) is finished at the end of this stage. The body is transparent due to the absence of muscular structure at beginning, but later on, myotomes are recognizable. The embryo completely turns itself (body reversal by positioning the head toward the vegetal pole) while the tip of the tail is still attached to the yolk sac. This is a critical step for further development to proceed. The body is attached to the yolk sac, while the tail detaches from the yolk toward the end of this stage and exhibits increasing tail movements. A tubular, pink-colored heart has been differentiated and begins to beat.

24.4.6 Embryonic Stage 6: Blood Formation (Figure 24.4f)

The head and tail of the embryo have distinctly separated from the yolk, which is reduced in its volume. The body length has increased distinctly. Transparent (later a light shade of pink) spherical blood cells and subsequently blood circulation can be observed. Pigmentation is prominent in the head, especially in the large eyes displaying brownish pigments, but less in the tail region. Skeletal muscles and myotomes become clearly visible.

24.4.7 Embryonic Stage 7: Remaining Organ and Fin Development (Figure 24.4g)

The head occupies one-third of the capsule space and has salient eyes with brown melanin pigmentation. The size of the entire embryo has increased substantially, with the tail reaching the posterior part of the eyes, and it displays continuous movement. The yolk sac becomes quite small, and yellow pigments start to appear on the trunk. Branchial arches with ventilating gills and opercula, a looped alimentary tract and jaws have developed. The fin folds have developed and are clearly visible.

24.4.8 Embryonic Stage 8: Hatching (Figure 24.4h)

A hindgut has formed, and the embryo fully occupies the capsule. The spinal cord is not flexed. The eyes are turning and silver shining (eyeshine from the tapetum). The embryo tries to hatch out: vigorous movements of the tail rupture an area close to the base of the eggshell (where the egg is attached to the substrate). The hatchlings emerge tail first, which usually takes place after sunset in complete darkness.

A relatively short larval development follows hatching and precedes metamorphosis. Even though developmental time frames for larvae are more variable than for embryos, the following studies have been combined to describe larval development and metamorphosis for anemonefishes in general: *A. ephippium* (Krishna 2018), *A. frenatus* (Putra et al. 2012), *A. nigripes* (Anil et al. 2012), *A. ocellaris* (Madhu et al. 2012; Roux et al. 2019b), *A. perideraion* (Salis et al. 2018a) and *A. sebae* (Gunasekaran et al. 2017).

24.4.9 Larval Stage 1: Preflexion of the Notochord (Figure 24.4i)

The larvae are mainly transparent, with some melanophores and xanthophores scattered over the head and body. Additionally, one or two horizontal lines of melanophores are present on the trunk, along the ventral midline. The embryonic fin folds remain undifferentiated and transparent. The notochord is still straight, in preflexion. Larvae are able to feed on live prey soon after hatching and process the food in a short, straight alimentary canal with the anus located in the middle of the body length. Stomach, midgut and hindgut are distinct, and the liver and pancreas are differentiated. The larvae display phototropic behavior and swim at the top of the water column.

24.4.10 Larval Stage 2: Flexion of the Notochord (Figure 24.4k)

The embryonic fin folds start to differentiate into the caudal, dorsal and anal fins, which exhibit first signs of soft rays. The notochord begins to flex by bending dorsally.

24.4.11 Larval Stage 3: Postflexion of the Notochord (Figure 24.4l)

The embryonic fin folds have completely differentiated into caudal, dorsal and anal fins. Both anal and dorsal fins exhibit the complete set of soft rays and spines that start to appear in a posterior–anterior gradient. The pelvic fins begin to differentiate. The notochord is in postflexion, resulting in a vertical position of the hypural bones. There are no major changes in pigmentation pattern or swimming behavior.

24.4.12 LARVAL STAGE 4: PELVIC SPINE (FIGURE 24.4M)

All fins, including the pelvic fins, are fully developed and possess all soft rays and spines. The numbers of melanophores and xanthophores scattered over the body are increasing. There is also a marked change in behavior, as larvae are not attracted to light anymore but swim close to the bottom. This can be considered the beginning of metamorphosis, which is accompanied by a shift from a pelagic to an epibenthic lifestyle.

24.4.13 LARVAL STAGE 5: APPEARANCE OF WHITE BANDS (FIGURE 24.4N)

During this stage, pigmentation patterns changes drastically. On one hand, chromatophores (bearing pigments, which shift from yellow to orange/red) are beginning to spread into the dorsal and anal fins as well as the caudal peduncle and head. On the other hand, the horizontal lines of melanophores start to disappear. Instead, the vertical white bands on the head and, depending on the species, on the body (*A. ephippium*, *A. frenatus*, *A. ocellaris*) start to emerge. They are transparent at the beginning but will adopt white color subsequently. Melanophores align at the border of the white bands. During metamorphosis, anemonefish larvae also undergo a rapid and extensive cranial remodeling that is linked with a change in preferred food items (Cooper et al. 2020). Furthermore, the shape of the body changes, and the width of the dorso-ventral axis increases, resulting in a more oval shape.

24.4.14 LARVAL STAGE 6: MATURATION OF ADULT COLOR PATTERN (FIGURE 24.4O)

Although the final maturation of the adult pigmentation is highly dependent on the anemonefish species, it is generally characterized by an increase in the thickness of the white bands. Pigmentation of the fins is completed during this stage in all species, with the caudal fin being the last to gain color. In *A. ocellaris*, for example, a third white band appears on the caudal peduncle after approximately 20 dph (days post-hatching), resulting in an adult that possesses three white bands. In *A. ephippium*, on the other hand, both the head and body white bands increase in thickness before they start to disappear. It has been described that this process starts with the middle portion of the body band at 50–55 dph and then slowly regresses toward the dorsal and ventral sites (completion by 160 dph). After that, the head band starts to disappear at approximately 240 dph and is completely gone by 300–310 dph. Similarly, larvae of *A. frenatus* exhibit a transient white band on the body at 20 dph, which subsequently disappears.

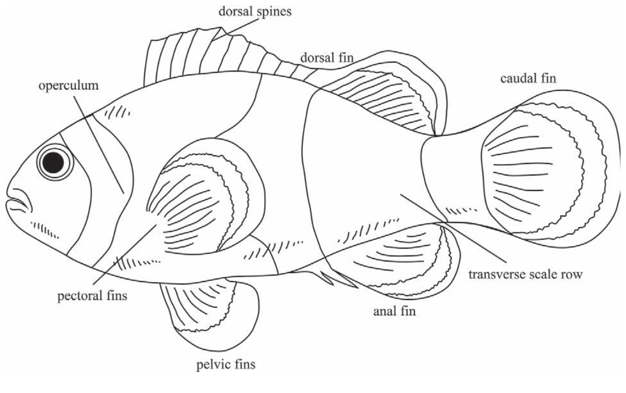

FIGURE 24.5 Schematic drawing of *A. ocellaris* showing external anatomical features.

24.5 ANATOMY

The following anatomical features can be used to distinguish members of the Amphiprioninae (Figure 24.5) from the remainder of the pomacentrids (Allen 1974; Nelson et al. 2016):

1. Nine to 11 dorsal spines
2. Suborbital, preopercle, opercle and interopercle bones with serrated or spinous margins and/or sculptured with radiating striae
3. Usually more than 50 transverse scale rows

Many tribe members also share the following features:

1. Teeth are uniserial and usually conical
2. Snout is mostly naked
3. Color pattern consists of one to three whitish bands on a darker background, which can be of various shades of orange, red, brown or black [exceptions are (i) *A. akallopisos*, *A. ephippium*, and *A. pacificus*, which do not have any bands, and (ii) *A. perideraion* and *A. sandaracinos*, which exhibit a dorsal stripe]

Anemonefishes are small sized (5–15 cm), and their body is oval and compressed (laterally thin) with a well-defined head and tail. As vertebrates, they possess all the characteristic organs and organ systems that specify this clade, such as a notochord, which develops into a vertebral column, gill arches, and neural crest cells. As representatives of the ray-finned fishes (Actinopterygii), the external anatomy is characterized by the presence of fin rays in the paired and unpaired fins, an operculum, a lateral line system and overlapping scales (Figure 24.5). Furthermore, they have specialized internal organs, such as three pairs of gill arches and a swim bladder.

The brains of anemonefishes exhibit typical features of teleostean brains; among others, these are: (i) large rhombencephalon; (ii) large unpaired cerebellum; (iii) two pronounced tectal halves located dorsal to the midbrain tegmentum and diencephalon; (iv) large, paired hypothalamic inferior lobe bulging out in the ventral brain surface; and (v) relatively small, everted telencephalon and relatively large olfactory bulbs (Nieuwenhuys et al. 1998). Furthermore, the visual system of *A. akindynos* was studied in high detail by

Sieb and colleagues (2019), who showed that retinal cones are arranged in a repetitive pattern, with four double cones surrounding a single cone.

All species of anemonefishes can produce and hear sounds, mainly composed of chirps and short and long pops (Parmentier et al. 2005; Parmentier et al. 2009). Pops are usually displayed as an aggressive, agonistic behavior against both conspecifics and heterospecifics. On the other hand, courtship sounds are more complex and differ in the number of pulses, pulse duration and dominant frequency. Sounds convey information about the size of the individual producing it, therefore implying the social rank of the emitter (Colleye et al. 2009). Sounds are produced by a series of cranial-focal interactions (Parmentier et al. 2007). First, the hyoid bar is lowered rapidly. Second, the sonic ligament, which connects the hyoid bar and internal parts of the mandible, is stretched and therefore forces the mandible to turn around its articulation, which in turn is closing the mouth. Third, the sound itself is made by collisions of the jaw teeth, with the jaw potentially acting as an amplifier. The sonic ligament represents a novel adaptation of the skeletal repertoire of anemonefish and other damselfish.

24.6 GENOMIC DATA

Actinopterygian fishes have a complex genomic history, and anemonefishes are of course no exception. In the 1970s, Susumu Ohno highlighted the importance of gene duplications as an important evolutionary mechanism that allows the creation of novelties during evolution (Ohno 1970). He further hypothesized that two rounds (2R) of whole genome duplications (WGDs) occurred early during vertebrate evolution. This was a controversial claim at the time, but it is now clear that there were effectively two genome duplications at the base of vertebrates. This is the famous “2R hypothesis”, which is now largely accepted even if there are still many discussions about the precise timing and even magnitude of these duplications (reviewed in Onimaru and Kuraku 2018).

In actinopterygians, the situation is even more complex, as a third genome duplication occurred at the base of the group (Meyer and Schartl 1999; Jaillon et al. 2004). This WGD is estimated to have taken place ca. 300 Mya and is often called the “teleost-specific genome duplication” or “Ts3R” (reviewed in Glasauer and Neuhauss 2014). Within teleosts, there were several more recent lineage-specific events, such as a fourth round of WGD in salmonids ca. 100 Mya (Berthelot et al. 2014) or in the lineage of carps within cyprinids ca. 5–10 Mya (Li et al. 2015). Anemonefishes are at the typical level of teleost fishes for which three WGDs have occurred: the two at the base of vertebrates, plus the one at the base of teleost fishes.

These events provide a higher complexity in terms of gene numbers in teleost fishes than in other vertebrate lineages such as birds or mammals. This may also be linked to the great number of species in teleosts as well as their extraordinary phenotypic diversity, although the link between WGDs and species diversity is still a matter of debate (Glasauer and Neuhauss 2014; Onimaru and Kuraku 2018).

The so-called DDC model (duplication-degeneration-complementation) predicts three possible outcomes following duplication of a gene: (i) non-functionalization (i.e. the loss of one of the duplicates), (ii) neo-functionalization (i.e. one of the copies retains the ancestral role, while the other duplicate assumes a novel functionality) or (iii) sub-functionalization (i.e. both duplicates assume a part of the function of the single ancestral gene). While the model predicts that the most likely outcome following duplication of a gene is the loss of one of the duplicates (i.e. non-functionalization), there are now several examples of neo-functionalization and sub-functionalization of duplicated genes (e.g. Kawaguchi et al. 2013 for stickleback hatching enzymes or Bertrand et al. 2004 for nuclear receptors in zebrafish).

This complex evolutionary history must be taken into account when the genome data of anemonefishes is analyzed. The genomic era of anemonefish research started in 2018 with the first complete genome, that of *A. ocellaris*, which was generated using a mix of nanopore and Illumina sequencing (Tan et al. 2018). The coverage of this genome was low (11X), but this allowed the prediction of around 27,000 genes and a genome size of 800 to 900 million base pairs (Mbp). Then, the genomes of *A. frenatus* (Marcionetti et al. 2018) and *A. percula* (Lehmann et al. 2019) followed, as well as a high-density genetic map of *A. bicinctus* (Casas et al. 2018). Genome size and gene number have been estimated to be of ca. 850 Mbp and 26,900 genes for *A. frenatus* and 908 Mb and 26,600 genes for *A. percula*. The *A. percula* genome, determined by using single molecule real-time Pacific Bioscience technology, was of exceptional quality, as the authors also performed Hi-C-based chromosome contact mapping, resulting in a genome assembly into 24 chromosomes (reviewed in Hotaling and Kelley 2019). This was in accordance with previous karyotypic studies done on *A. perideraion* (Supiwong et al. 2015). This *A. percula* genome is now a unique resource for the whole community. Another major achievement was the genome assembly and annotation of nine species of anemonefish (*A. akallopisos, A. bicinctus, A. melanopus, A. nigripes, A. ocellaris, A. perideraion, A. polymnus, A. sebae* and *P. biaculeatus*) and a related damselfish outgroup, allowing for the first time insights into the genomics of anemonefish radiation and identification of genes that may be implicated in the symbiosis with sea anemones (Marcionetti et al. 2019). These datasets have already been used by independent authors to analyze specific gene sets such as peptidic hormones (Southey et al. 2020). Certainly, this is only the beginning of the anemonefish genomic era. We can anticipate that soon the genomes of all 30 known species of anemonefish will be available. Several genomes of distinct populations of anemonefishes are currently being sequenced, thus opening the way to population genomic analysis of these iconic fishes.

Complete genome sequences have been complemented by several transcriptomic data sets that started to tackle specific questions. A transcriptome of *A. ocellaris* post-embryonic development, spanning newly hatched larvae until settled juveniles, has been determined (Roux et al. in preparation). Another area of interest is the identification of genes related to the differently colored areas (white, orange and black) of *A. ocellaris* (Maytin et al. 2018; Salis et al. 2019a). This, combined with detailed pharmacological and microscopic analysis, has allowed researchers to determine that iridophores are responsible for the white color in this species but also to identify new iridophore and xanthophore genes in fish (Salis et al. 2019a, reviewed in Irion and Nüsslein-Volhard 2019; Patterson and Parichy 2019). Transcriptomic analysis has also been applied to the spectacular sex change abilities of anemonefishes. For example, a study of *A. bicinctus* from the Red Sea has revealed a complex genomic response in the brain and subsequently in the gonads with a prominent effect on genes implicated in steroidogenesis (Casas et al. 2016). Genes implicated in reproduction have also been studied in *A. ocellaris* (Yang et al. 2019).

Last, transcriptome analysis was used in the context of aging, as anemonefishes are known to have a long lifespan (Sahm et al. 2019). The authors have detected positively selected genes in *A. clarkii* and *A. percula* and tested if these genes were similar to those found in other models of aging such as mole rats or short-lived killifishes. They concluded that molecular convergence is likely to occur in the evolution of lifespan.

These examples are in fact the exhaustive list of genomic and transcriptomic studies done so far on anemonefishes. Due to low-cost high-throughput sequencing, it is likely that this will increase exponentially in the coming years as these fishes will be used more and more as experimental models which allow to link ecological, evolutionary and developmental studies.

24.7 FUNCTIONAL APPROACHES: TOOLS FOR MOLECULAR AND CELLULAR ANALYSIS

24.7.1 Husbandry

Generally, the success of an emerging model species is linked to a feasible husbandry as well as the ease of obtaining samples. For marine teleosts, this can pose difficulties, as it might be difficult to achieve reproduction in captivity or to reliably locate them in the natural environment. Anemonefishes provide an excellent model for both scenarios. On the one hand, due to their close association with sea anemones, researchers are able to locate and re-locate anemonefishes with relative ease in the wild, enabling them to conduct long-term experiments with the same individuals. On the other hand, they are very well adapted for captive life, having been in the hobbyist trade for decades. For tropical marine fishes, anemonefishes are relatively tolerant to temperature (24°C to 28°C) and salinity variations (25 to 40‰) (Dhaneesh et al. 2012). Smaller species, like *A. ocellaris*, *A. percula* and *A. sandaracinos*, can be kept in 60-L tanks, while bigger species, such as *A. clarkii*, *A. frenatus* and *P. biaculetatus*, will need up to 200-L tanks. In captivity, anemonefishes thrive without the addition of sea anemones and establish breeding pairs, which usually reproduce all year around. Both partners will participate in selection of an appropriate substrate and its cleaning, usually a terra cotta pot, ceramic tiles or even the glass walls. Egg clutch sizes vary greatly between and within species and depend on previous reproductive experience, nutrition and body size. A sufficient amount of eggs can be obtained for experimental purposes (up to 700–1,000 eggs) every 14–21 days. For experiments that require embryonic stages (such as micro-injection), the eggs can be scraped off substrate (for example, with a razor blade) and can be transferred to an egg tumbler or petri dishes for incubation. For experiments that require larval stages, the eggs remain with the parents until they are supposed to hatch (night of hatching). For hatching, they can be transferred into a separate aquarium by replacing the substrate with the attached eggs. Alternatively, if external water circulation can be interrupted, the larvae can hatch in the parent's aquarium and subsequently be transferred to a different aquarium by attracting them with a light source. This, however, is only advisable if there is no sea anemone in the same aquarium. Larvae can either be raised in small aquaria (20–30 L) or in 500–1,000-mL beakers (containing 1–20 larvae per beaker; Roux et al.). They are first fed with a mixture of micro algae and rotifers and later on *Artemia nauplii*. Juveniles are also fed with *Artemia nauplii* and either powdered food or food pellets (depending on size). The diet of adult fish is diverse and can be adjusted easily: *Artemia*, food pellets, chopped mussels, squid, shrimp and egg yolk, as well as vitamin supplements (Anil et al. 2012).

Several standard approaches have been successfully established in anemonefishes, and only a few will be highlighted here.

24.7.2 *In Situ* Hybridization

In situ hybridization is a very powerful tool to study temporal and spatial requirements of specific genes in their cellular context. In *A. frenatus*, embryonic mesodermal and neuroectodermal development has been followed by gene expression analysis of *no tail* (*ntl*) and *sox3*, respectively (Ghosh et al. 2009). Further, a comparative expression analysis of *orthodenticle homeobox 2* (*otx2*) in the olfactory placode of larval *A. percula* indicates that this gene is required for olfactory responses to settlement cues (Veilleux et al. 2013). Moreover, *in situ* hybridization can validate results acquired employing alternative approaches, such as transcriptomics. For example, a recent study revealed several upregulated genes in the white skin of *A. ocellaris*, some of which could be confirmed via *in situ* hybridization on juvenile skin sections (Salis et al. 2019a). Fluorescent *in situ* hybridization (FISH) has also been successfully established in anemonefishes. In *A. akindynos*, it has been shown that long wavelength-sensitive (LWS)-related opsin genes are exclusively expressed in double cones, while short wavelength-sensitive (SWS)-related opsins are only expressed in the interspaced single cones (Stieb et al. 2019).

24.7.3 Immunoassay

Commercial enzyme immunoassay (EIA) kits are available to analyze biochemical aspects of cells, such as hormones, neurotransmitters and second messenger molecules (such as cAMP). In 2010, Mills and colleagues validated two such kits for measuring 11-ketotestosterone and cortisol concentration, respectively, using blood plasma from *A. chrysopterus* and *A. percula*. They found that a minimum of 5–7 µL blood plasma is sufficient to confidently estimate steroid hormone concentrations, which is especially valuable when working in the field. Other hormones, such as thyroid hormones, can be routinely measured using phenobarbital extraction and ELISA detection according to the method developed by Kawakami et al. (2008) and Holzer et al. (2017).

24.7.4 Use of Drugs for Functional Experiments

Pharmacological reagents/small molecules have been used widely in zebrafish, *Danio rerio*, and helped to broaden our understanding of zebrafish biology. To date, only few of them have been tested in anemonefishes, but they pose a great potential in a variety of fields. For example, it has been shown that the small molecule TAE 684 inhibits *Alk* and *Ltk* dependent iridophores in zebrafish (Rodrigues et al. 2012). In *A. ocellaris*, TAE 684 treatment of larvae results in juveniles without white bands, thus providing evidence that iridiophores are responsible for the white color of anemonefishes (Salis et al. 2019a). Furthermore, treatment with BMP inhibitors, such as dorsomorphin or DMH1, in early embryonic stages can result in dorsalization in zebrafish (Yu et al. 2008) and *A. ocellaris* (M. Klann personal observations) alike.

24.7.5 Cell Culture

So far, there is only one report on cell culture from anemonefish explants, even though this technique is extremely valuable for research projects focusing, for example, on virology, cytobiology and oncology/disease, but also for environmental toxicology/ecotoxicology or genetics/genomics. Patkaew and colleagues (2014) used *A. ocellaris* vertebrae explants to establish a corresponding primary culture. Four days after the initial implantation, fibroblastic cells could be seen, which then multiplied rapidly, reaching 70–80% confluence within four to five days. The fifth passage was preserved in liquid nitrogen for one month and subsequently assessed. The average viability after thawing and seeding has been reported with 80%, with a 57% cell recovery and no obvious changes in cell morphology or growth pattern. Even though they do not give details, the authors also state that the employed explant method (without the use of enzymes) resulted in successful primary cultures from gills, skin and vertebrae from other anemonefishes.

24.7.6 Genetic Markers

Genetic markers, particularly microsatellites, have been developed and are now available for several anemonefish species. They are widely used to study population genetics and have been used for example to investigate phylogeographic connectivity (Dohna et al. 2015), detect and monitor hybridization events (He et al. 2019; Gainsford et al. 2020), elucidate self-recruitment of larval dispersal (Jones et al. 2005), estimate connectivity between marine protected areas (MPAs) (Planes et al. 2009) and even to determine the composition of social groups (Buston et al. 2007). A substantial number of population genetic and dynamic studies have been done on *A. percula* populations of Kimbe Bay (Papua New Guinea), with the notable construction of the first multigenerational pedigree for a marine fish population (Salles et al. 2016). Such genealogy provides an opportunity to investigate how maternal effect, environment or even philopatry can shape wild fish populations (Salles et al. 2020). Probably due to its localization in the diversity center of anemonefishes, Kimbe Bay represents a privileged study site for the investigation and testing of numerous ecological and evolutionary theories and mechanisms. For example, a recent study demonstrated that the combination of ecological and social pressure promotes the evolution of non-breeding strategies (Branconi et al. 2020). The integration of the generated data provides an invaluable cornerstone for future studies in the general field of ecology and evolution.

24.8 CHALLENGING QUESTIONS, BOTH IN ACADEMIC AND APPLIED RESEARCH

Anemonefishes are ideal emerging model systems to answer a wide range of questions in biology, including but not limited to conservation, host recognition, evolutionary mechanisms and biomedical research. Missing functional approaches are also discussed at the end of this section.

24.8.1 Human Impact and Conservation

Anemonefishes live in coral reefs, which are among the most threatened ecosystems. Many anthropogenic stressors act either globally or at a local scale: global warming, pollution, ocean acidification and deoxygenation, to name just a few (Altieri et al. 2017; Albright et al. 2018; Hughes et al. 2018; Porter et al. 2018). The effects of stressors on coral reef fishes can be studied at different levels, including growth, physiology, development, genetics, bioaccumulation and behavior. Information gained in any of these fields will provide a better understanding of the coral reef ecosystem and ultimately, its conservation. A few exploratory studies investigating the effect of anthropogenic stressors on anemonefishes have already been conducted, and some will be introduced subsequently. A chemical compound found in sunscreens acting as a UV filter (benzophenone-3) perturbed feeding and swimming behavior and led to a decrease of body weight even at small concentrations of 1 mg/l (Chen et al. 2018; Barone et al. 2019), whereas higher concentrations of 100 mg/l resulted in 25% increased mortality rate (Barone et al. 2019). The direct impact of global warming (increased water temperature) on the physiology of anemonefishes has been investigated. The cellular stress responses (quantification of molecular biomarkers) of adults raised for one month at 26°C (control) or 30°C (elevated temperature) have been compared, and tissue-specific differences could be found, with muscles, gills and liver being the most reactive tissues (Madeira et al. 2016). The authors concluded that if individuals are not able to adapt to elevated temperatures, lower reproductive success, reduced growth and disease resistance would most likely occur (Madeira et al. 2016). Sea anemone bleaching (loss of symbiotic zooxanthellae) poses an important indirect effect of global warming for anemonefishes. It has been shown that juveniles of *A. chrysopterus* living in bleached sea anemones (*H. magnifica*) had an increased standard metabolic rate (up to 8%) when compared to juveniles from unbleached sea anemones (Norin et al. 2018). The authors suggested that this increased minimum cost of living might result in reduced fitness (revised energy allocation) such as reduced growth rate, spawning frequency or lower fecundity. In the same species, it has been shown that fish living in bleached hosts experienced changes in stress and reproductive hormones (cortisol and 11-KT and 17β-estradiol, respectively) (Beldade et al. 2017). Spawning frequency and clutch sizes were lower than in unbleached hosts (respectively, 51% and 64%), while egg mortality was higher (38%), leading to an overall fecundity decrease of 73%. However, after host recovery, all hormonal and reproductive parameters went back to their pre-bleaching levels. This strongly suggests a key role of hormonal response plasticity in fish acclimation to climate changes (Beldade et al. 2017). Similarly, a decrease in egg production in bleached anemone has been reported for *A. polymnus* (Saenz-Agudelo et al. 2011). None of the previously mentioned studies reported mortality of adult fish subsequent to a bleaching event. However, by following two consecutive bleaching events, Hayashi and Reimer (2020) showed that host anemones took longer to recover after the second bleaching and that one individual even completely disappeared, together with the anemonefish pair living in it. This study indicates that if temperature abnormalities are to happen regularly, sea anemone resilience to bleaching might be impaired, which can have direct consequences for anemonefishes. Another indirect effect of global warming is ocean acidification. Indeed, when reared under simulated ocean acidification conditions, olfactory and auditory abilities of anemonefish larvae were disrupted, which usually provide important cues to locate the reef and their hosts (Munday et al. 2008; Dixson et al. 2010; Simpson et al. 2011; Holmberg et al. 2019). Noise induced by humans is classified as a form of pollution. Indeed, a study showed that embryos of *A. melanopus* reared under the influence of playback boat noise exhibited faster heart rates (about 10% increase of cardiovascular activity) than ambient reef controls (Fakan and McCormick 2019). Although survival rates of embryos subjected to noise did not change, it is possible that embryogenesis is nevertheless negatively affected, leading to larvae and juveniles with reduced fitness (Fakan and McCormick 2019). Besides boat noise, anemonefishes can also be directly affected by other recreational activities such as scuba diving. Indeed, divers tend to approach these iconic fishes as closely as possible, but this human attitude could induce changes in the behavior and stress level of the fish (Hayashi et al. 2019a). In the long run, repeated human presence could affect anemonefish fitness by impairing essential behaviors such as courtship, egg care and feeding (Nanninga et al. 2017). Another drawback of their popularity is that anemonefishes are highly targeted by the aquarium trade. Indeed, the same attributes that make them good model organisms attract aquarists (longevity and exotic symbiosis) and permit easy harvesting in their natural environment (Shuman et al. 2005). Pomacentrids represent around 76% of wild-caught ornamental fish imported in the United States, with *A. percula* and *A ocellaris* in fifth place (after four species of damselfish) (Rhyne et al. 2012), even though they can be captive-bred easily. Anemonefishes represent up to 57% of all collected organisms in the Philippines (Shuman et al. 2005). There, exploited sites exhibit lower anemonefish biomass than protected sites, and fish size distribution tends to be skewed toward small fish. For *A. clarkii*, even the number of individuals present in exploited sites was lower, and similar results were observed for the anemone *H. crispa* (Shuman et al. 2005). Those results reflect the non-negligible impact of aquarium trade on anemonefishes and host anemone populations.

Another human impact that has been studied is coastline anthropization. Recent studies showed that it could not only lead to low replenishment rates but also affect community structures and diversity of anemonefishes (Hayashi et al. 2019b; Hayashi et al. 2020).

While many aspects of anemonefishes biology and ecology have been studied, very little has been done to integrate those findings in applied fields such as conservation biology (but see Planes et al. 2009; Hayashi et al. 2019b, 2020), which, in the actual context of ever-growing human pressures, should be one of the priorities of the research community.

24.8.2 Host Recognition and Settlement Clues

Numerous studies have focused on the symbiotic relationship between anemonefishes and their host anemones, with the aim to understand how juvenile recruitment occurs. Although it is well documented that anemonefishes can distinguish different host anemones and their health status (bleached vs. unbleached) using chemical cues (Murata et al. 1986; Arvedlund and Nielsen 1996; Arvedlund et al. 1999; Miyagawa-Kohshima et al. 2014; Scott and Dixson 2016), composition and structure of these chemicals still remain unknown. A study found an upregulation of *otx2* expression, a transcription factor frequently associated with olfactory imprinting, in larvae which were exposed to settlement odors compared with no-odor control larvae of *A. percula* (Veilleux et al. 2013). This chemical imprinting is believed to occur during late embryonic development and the first hours after hatching and is sufficient to recognize all species-specific partner host anemones regardless of the parents' host anemone (Arvedlund et al. 2000; Miyagawa-Kohshima et al. 2014). However, it has also been shown that anemonefishes possess a limited innate recognition

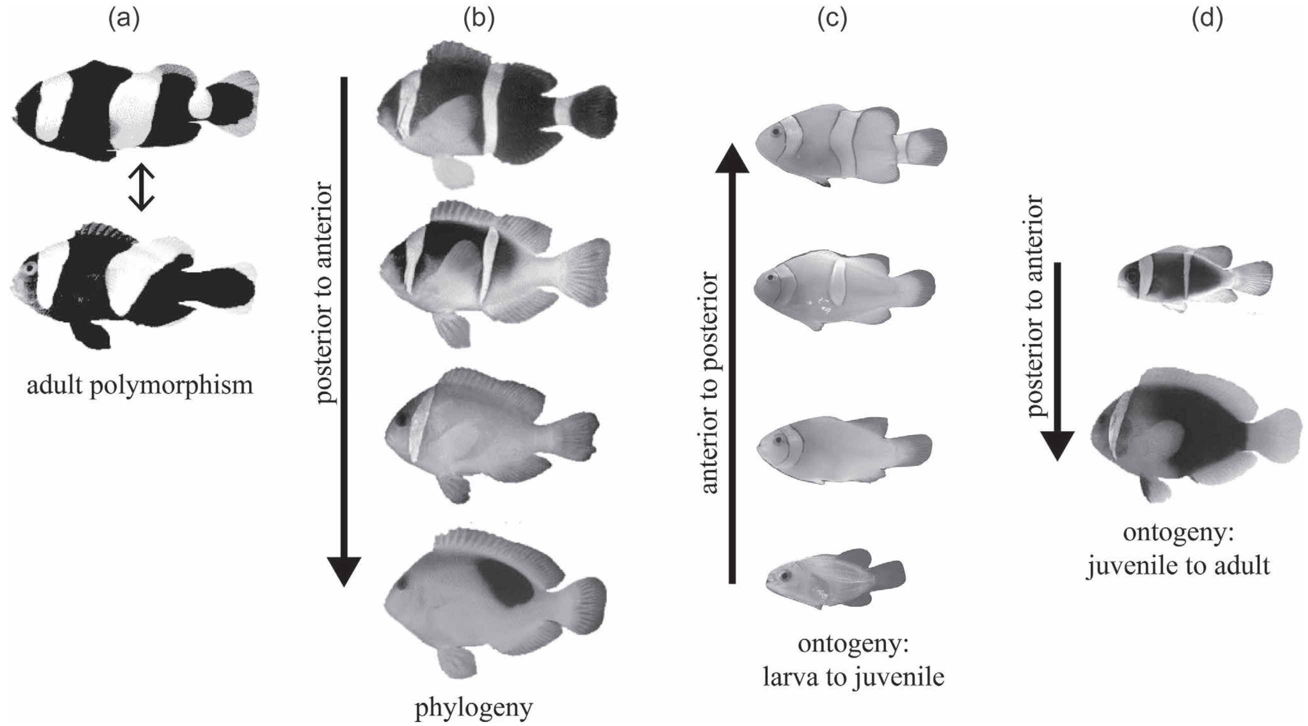

FIGURE 24.6 Evolutionary and developmental white band acquisition. Opposing trends have been described, but the underling mechanisms remain unsolved.

of partner and non-partner host anemones (Miyagawa-Kohshima et al. 2014). Field experiments further showed that new recruits do not discriminate between occupied and unoccupied host anemones (Elliott et al. 1995) but did encounter highly aggressive behavior from the resident fish (especially resident juveniles). Usually the new recruit would cease approaching an inhabited host after several aggressive interactions and try to locate a different host (Elliott et al. 1995). This eviction of juvenile anemonefishes has been widely noted and is believed to be the reason for the formation of sub-symbiotic partnerships if symbiotic partnership cannot be established (i.e. use of a sea anemone species that is not preferred) (Miyagawa-Kohshima et al. 2014). Most studies on anemonefish settlement have focused on the cues involved when selecting a host anemone, but cues to settle out of the plankton into the benthic reef habitat are less well investigated. They are unlikely to be the same, as it has been shown that chemical cues from anemones can only guide juveniles if they are relatively close to and downstream of an anemone (typically 2 m, with a maximum around 8 m) (Elliott et al. 1995). Due to the relative ease of obtaining naive larvae (i.e. aquarium-raised without sea anemone contact), field experiments can be conducted to validate experimental hypotheses. Once we have a better understanding of anemonefish settlement, we will be able to investigate how other coral reef fish larvae select nurseries and/or microhabitats. Selection of an appropriate substrate is of great importance for young fish, as it will ultimately determine their survival and breeding success.

24.8.3 EVOLUTIONARY MECHANISMS

Anemonefish phylogeny has been used to investigate how hybridization and species diversification are linked (Litsios and Salamin 2014). This phylogeny was also used to compared the evolution rate of anemonefishes at both intra- and interspecific scales (i.e. micro- and macro- evolution) (Rolland et al. 2018). Other new approaches, such as quantitative genetics, might also provide a better understanding of evolutionary mechanisms. This kind of approach assesses how phenotypes are shaped given the relatedness between individuals sharing similar traits and the environment in which they are living (Thomson et al. 2018). For example, Salles et al. (2020) estimated the proportion of variance in lifetime reproductive success (LRS) explained by genetic and environmental factors. When compared to environment, genetics play a minor role, resulting in low heritability and evolvability. This suggests that in its current state, the population potential for evolutionary change is very limited, highlighting the importance of plasticity to enable rapid adaptive responses. Another complex feature observed in anemonefishes is color polymorphism, which has been noted to occur at multiple scales, with melanization being the predominant one (see Figure 24.1 for an example in *A. clarkii*). Geographical variation in coloration is common among widely distributed species, but sympatric variations have also been reported in populations in which sexual dichromatism and ontogenetic differences govern pigmentation (Moyer 1976; Fautin and Allen 1997). A suite of interacting and conditional ecological factors encompassing social rank, host anemone species and location had been identified as the primary factors predicting distribution of melanistic morphs (Militz et al. 2016). However, phylogenetic studies on melanistic *A. clarkii* showed that specimens cluster by color rather than geographical origin: a melanistic specimen from Bali is more closely related to another melanistic individual originating from Papua New Guinea than to a syntopic orange *A. clarkii* (Litsios et al. 2014a). Another common polymorphic feature of anemonefish color pattern is the variation of band number, regularly observed in *A. clarkii, A. melanopus* and *A. plolymnus* (Figure 24.6a). This suggests complex mechanisms might be involved in anemonefish polymorphism. Salis and colleagues (2018b) mapped the occurrence and number of bands on the phylogeny to reconstruct the ancestral state and could show that the diversification of anemonefish color pattern results from successive caudal to rostral losses of bands during evolution (Figure 24.6b). This is in contrast with the developmental acquisition of bands, which appear in an anterior to posterior gradient (Figure 24.6c). Interestingly, juveniles of some species have supplementary bands that disappear later caudorostrally (Figure 24.6d). The reduction of band number during ontogeny matches the sequence of band loss during evolution, demonstrating that diversification in color pattern among anemonefish lineages resulted from changes in developmental processes. The functional aspect of anemonefish skin color and pattern remains unclear. However, it has been suggested that color patterns may (i) be used in advertising social rank (Fautin and Allen 1997; Militz et al. 2016), (ii) signal individual identity (Fricke 1973; Buston 2003a), (iii) provide disruptive coloration (Salis et al. 2018b) and (iv) be used for species recognition (Salis et al. 2018b; Salis et al. 2019b). Yet developmental mechanisms underlying the color pattern formation have still not been identified. However, a Turing-like model (that patterns zebrafish or angelfish, for example) cannot explain the appearance and/or disappearance of bands during ontogeny, thus suggesting that band formation is controlled by specific patterning mechanisms that remain to be analyzed. The dorsal fin might act as a spatial reference, since its size and geometry have been significantly correlated with the number of white bands (Salis et al. 2018b). Given the increase in interdisciplinary studies, considerable improvement in the understanding of evolutionary mechanisms should be expected in the coming years.

24.8.4 BIOMEDICAL RESEARCH

Anemonefishes are a promising model system for biomedical research, even though studies in this field are limited so far. On one hand, they have a relatively long life span and, on the other hand, their ability to avoid nematocyst discharge is rare among vertebrates. Anemonefishes are one of a few species that offer the opportunity to study longevity and aging. Indeed, they have a long life expectancy, which is approximately six times longer than that predicted for other small fish (Buston and García 2007; Sahm et al. 2019), and they reproduce monthly all year around. Using anemonefish, a recent study (Sahm et al. 2019) suggested that the mitonuclear balance (i.e. balance between expression of nuclear and mitochondrially encoded mitochondrial proteins) plays a key role in aging, which opens the gate to explore those genetic pathways involved.

Although many studies have attempted to unveil how anemonefishes avoid the negative effects of nematocyst stinging, there are still many open questions and various competing hypotheses (see Section 24.3). Indeed, a field study with several species of anemonefish showed that new naive recruits (around 20 dph) are able to enter their host anemones without being harmed on the first attempt (Elliott et al. 1995). Occasionally, the new recruits adhered to the tentacle but usually could break free and, after a short acclimation process, could enter unharmed. From a biomedical standpoint, it is of great interest, as understanding how anemonefishes avoid being stung by the hosts' nematocysts might lay a foundation for possible prevention and therapy of negative human interactions with jellyfish, for example. Additionally and rather unexpectedly, the anemonefish queuing system has been used to serve as the basis of a novel brain tumor segmentation algorithm (Mc and Subramanian 2016).

24.8.5 MISSING FUNCTIONAL APPROACHES

Casas et al. (2016) performed the first *de novo* transcriptome analysis of wild *A. bicinctus* and highlighted the rapid and complex genomic responses of the brain during sex change, which is subsequently transmitted to the gonads. This transcriptomic data (Casas et al. 2016; Yang et al. 2019) will broaden our understanding not only of the physiological mechanisms involved but also of the perception and processing of external cues into a coordinated response that characterizes sex change (Lamm et al. 2015; Liu et al. 2017). Advances in molecular endocrinology, genomic and transcriptomic data in anemonefishes will allow opening new avenues in our understanding of sex change and sex determination in fishes and more widely in vertebrates. Moreover, extensive efforts have been put in by several research groups to establish micro-injection (Roux et al. 2020) and associated genome editing, such as CRISPR/Cas9 in anemonefishes (Mitchell et al. 2020). This is a much-needed toolkit to gain functional data and will be applicable to a range of research areas. Micro-injection is possible, yet mortality rates are still high, and obtaining larvae remains difficult (Mitchell et al. 2020; Roux et al. 2020). However, once established, the possibility of modifying specific genetic aspects will advance the field of anemonefish research, as well as research on coral reef fish, immensely. Although there are several pet shop mutants available with diverse color patterns, the underlying mutations and exact mechanisms have not been studied in detail.

24.9 CONCLUSION

This chapter summarizes the past and most recent research finding as well as future perspectives, revealing the great potential anemonefishes offer as emerging marine fish models. Future research on anemonefishes will complement studies on traditional model organisms in a wide variety of biological areas, from pigmentation to neurobiology. Their unique biological attributes open perspectives to tackle new questions related to aging, sexual differentiation, symbiosis, growth or even social organization. Anemonefishes have and will always remain prominent models for ecological studies, but now those can be linked with lab based evo-devo approaches, which is hardly possible with other model organisms. As there is a lack of convenient experimental models for marine fishes, we hope and strongly believe that this model will find its place in the vast array of new models available for the biologists of tomorrow.

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