8 Age and Longevity

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CONTENTS

8.1 INTRODUCTION

8.1.1 SENESCENCE IN TELEOSTS

Teleost fishes are a large and diversified class of vertebrates comprising more than 20,000 existing species (Patnaik et al. 1994). Within this biodiversity, some species (zebrafish, killifish, and clownfish; Schartl 2014) show some interesting traits that make them ideal model species for ecological, physiological, genetic, and biotechnological investigations (Holtze et al. 2021; Mutalipassi 2019; Bahls et al. 2003; Levy and Currie 2015; Bodnar 2016; Goldstein and King 2017). Among these aquatic models, some species occupy the extremes in the spectrum of ageing processes, with lifespan differences of two orders of magnitude. Some examples include short lifespan species, such as the turquoise killifish *Nothobranchius furzeri* which presents a lifespan of few months (Cellerino et al. 2015; Terzibasi et al. 2007), and species with a life expectancy of more than a century, as in the case of some rockfishes of the genus *Sebates* (Mangel et al. 2007) and the Greenland shark *Somniosus microcephalus* (Nielsen et al. 2016). In teleost fishes, we can identify three types of senescence (Finch 1998). The first is the rapid senescence, generally linked with the sudden death at first spawning, with examples in species such as lamprey and salmon. Rapid senescence is usually triggered by hormonal inductions (Finch 1998). The second one is the gradual senescence characterized by an age-related decline in reproduction, loss of compact bone, endothelial proliferation, collagen oxidation, and accumulation of brain amyloid and other forms of protein aggregation, etc. (Kishi 2004). This second typology of senescence has been observed in many other vertebrates including humans, and it is present in teleosts such as *Poecilia* spp. (Reznick et al. 2006) or zebrafish (Kishi et al. 2003). The third typology of senescence is observed in fishes characterized by an indeterminate growth, where senescence is supposed to be slow or negligible, with evidence of undiminished functions during ageing and with reproductive activities maintained in old age (Finch 1998).

8.1.2 THEORIES CONCERNING THE EVOLUTION OF SENESCENCE

Evolutionary theories of ageing correlate low extrinsic mortality conditions to the evolution of slow senescence and increased lifespan. Extrinsic mortality is one of the most important factors which contribute to the accumulation of deleterious mutations by limiting the exposure of the late-acting mutation to selection. It has been theorized that extrinsic mortality is the principal determinant of the senescence rate in age-structured populations (Kirkwood 2000). Three main theories were proposed to explain the evolution of ageing. The mutation accumulation theory postulates that ageing is caused by the accumulation of mutations with late-life phenotypes that behave like quasi-neutral mutations as they have marginal effects on whole-life fitness (Medawar 1952); the antagonistic pleiotropy theory postulates that alleles that are positively selected due to their effects on growth and fertility early in life reduce fitness later in life (Williams 1957); and the disposable soma theory postulates that ageing is caused by the trade-off in the energetic resources devoted to growth and maintenance (Kirkwood 2002). All three theories have as a corollary that reduced extrinsic mortality should drive the

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evolution of longevity as observed by the exceptional longevity of several vertebrate species living in predator-free or protected environments, such as caves (Voituron et al. 2011) or arboreal habitats (Shattuck and Williams 2010), or in species that evolved the ability to produce or bioaccumulate antipredator chemical compounds, as in the case of several lineages of amphibians and snakes (Hossie et al. 2013).

The aforementioned killifish *Nothobranchius furzeri* is a case study that provides an example of relationships between extrinsic mortality and the evolution of senescence. *Nothobranchius* species, characterized by accelerated senescence and short lifespan, are subjected to high extrinsic mortality (Terzibasi et al. 2013; Blažek et al. 2017; Cellerino et al. 2015) and this selection revealed a correlation between the evolution of mitochondrial biogenesis genes and lifespan (Sahm et al. 2017). In *N. furzeri*, investigations on positively selected genes, within three evolutionary lineages, demonstrated that genes under positive selection were significantly enriched for functions involved in all steps of mitochondrial biogenesis, as in the case of mitochondrial proteins and respiratory chain complex I (Sahm and Cellerino 2017). Under the influence of such a peculiar ecology, protein expression has evolved to sustain fast growth and early maturation. Yet, through the process of antagonistic pleiotropy, they drive at the same time an accelerated ageing process (Sahm and Cellerino 2017). For these reasons, *N. furzeri* is one of the most interesting and emerging model species for ageing studies, due to its unique short life expectancy which is extremely reduced in those strains living in the most arid regions of its distribution area (Terzibasi et al. 2008; Holtze et al. 2021).

8.1.3 Searching for a New Long-Lived Model Organism

In contrast to *Nothobranchius* which is widely used as an experimental model species in ageing for its lifespan of less than one year, the research community still lacks an established model organism with exceptional longevity that can be easily cultured in captivity. Current field models are inadequate for common search purposes because of various difficulties related to their culturing. The scientific community is asking for a model that can be easy to rear and manage and that can, at the same time, answer scientific questions, for example in the ageing research field. For instance, the Greenland shark (*Somniosus microcephalus*), a model species for field studies, is quite complex to culture and breed in captivity due to its size, the unusual and remote habitat, and the extremely slow generation time. In addition, further studies are needed to explore and take advantage of the full potential of these remote and/or poorly studied species. Especially in comparative studies dealing with the ageing field, it is needed to improve our knowledge not only of transcriptome and genome sequences of these target species, but also of their distinctive physiology (Holtze et al. 2021). Several other species that demonstrated extreme lifespan, such as the olm (*Proteus anguinus*), an aquatic cave-dwelling salamander, are rare in nature and exhibited undesirable features, such as an age at sexual maturity of 15.6 years and the fact that they lay, on average, 35 eggs every 12.5 years (Voituron et al. 2011), making them not suitable for laboratory investigations. Similar problems are faced when trying to use long-lived mammals that have been demonstrated to be difficult or impossible to be kept in captivity and manipulated experimentally (Sahm et al. 2019; Holtze et al. 2021). These constraints create the necessity for a long-lived vertebrate that has all the characteristics required in a model organism (Ankeny and Leonelli 2021).

8.2 ANEMONEFISHES: IDEAL MODELS OF LONGEVITY?

8.2.1 Anemonefish: The Answer to the Search for a New Marine Model

Fishes belonging to the subfamily of Amphiprioninae (Pomacentridae family) could provide such a model. Amphiprioninae comprises two genera, *Amphiprion* and the monospecific *Premnas*, and all the species in these two genera are commonly known as anemonefish or clownfish. Clownfishes evolved a peculiar adaptation, probably inherited by a common ancestor, that enables a symbiosis with sea anemones and this symbiosis can be considered iconic of coral reefs. These species, originally used only in ecological investigations, are gaining interest as a more flexible model with potential application in various research fields. Consequently, the interest in clownfishes as model organisms increased in the last decade, for example in ageing (Sahm et al. 2019) and eco-evo-devo (Roux et al. 2020) studies, with several publications that described the unique and distinctive characteristics as well as the advantages of this model compared to the standard ones. They possess several characteristics that are precious in model organisms that make. They are phylogenetically related to damselfishes, such as *Chromis* and *Dascyllus* (subfamily Chrominae), *Chrysiptera* (subfamily Pomacentrinae), and *Lepidozygus* (subfamily Lepidozyginae) (Quenouille, Bermingham, and Planes 2004). The complex phylogeny of anemonefishes has been resolved using mtDNA and nuclear markers, as well as whole-genome sequencing (Litsios et al. 2012; Marcionetti et al. 2019).

8.2.2 Anemonefish Peculiarities

Clownfish species are socially controlled sequential protandrous hermaphrodites (Olivotto and Geffroy 2017) and their assemblages are characterized by a strong social hierarchy based on size that behaves as queues for reproduction (Casas et al. 2016). In fact, the two largest individuals are the dominant female and dominant male, respectively; this breeding couple is surrounded by a variable number of immature males of smaller size (Fricke and Fricke 1977). Apart from the aforementioned physiological and genetic peculiarities, clownfishes are relatively small, with some species that achieve a maximum size of less than ten centimetres. In addition, several *Amphiprion* species are cheap, common as well as robust aquarium fishes, easy to culture, feed, and breed in large numbers (Roux et al. 2021). All these characteristics allow the use of these species and in particular the false anemonefish *Amphiprion ocellaris* or the orange clownfish *Amphiprion percula*, to investigate a wide range of scientific questions in field or mesocosms, ranging from sex changes to social behaviors, sound production, as well as the symbiotic relationship with sea anemones (Marcionetti et al. 2019; Litsios et al. 2012; Dixson et al. 2014; Mebs 2009; Buston 2003).

8.2.3 Anemonefish and Predation

The search for long-lived vertebrates focused on those species that live in predator-free environments, or in stable habitats being two of the aforementioned environmental constraints correlated with the evolution of extreme lifespan (Wilkinson and South 2002; Rose 1991). Anemonefish belongs to this group of species that occupy a predator-free environment thanks to the symbiosis with sea anemones that provides for protection from predation. *Amphiprion* fishes are not hit by the lethal nematocysts present in the epithelium of the sea anemones tentacles thanks to a protective mucous coat that prevents the discharge of these cnidarian organelles (Mebs 2009). When facing danger these small reef fishes instantly search for protection in anemones' tentacles and it has been proven that if deprived of their symbiont anemone, the predation rate on clownfishes drastically increases (Elliott, Elliott, and Mariscal 1995; Mariscal 1970). Field observations have demonstrated that intimate relationships with anthozoans are not unique to clownfishes but can be found in other species of Pomacentridae, such as *Chromis viridis*, that showed interesting relationships with scleractinian corals (Ben-Tzvi et al. 2008; Lecchini et al. 2006). These species use branching corals as shelters (Garcia-Herrera et al. 2017; Holbrook et al. 2008), and the lack of available refuges exposes them to high predation (Hixon et al. 1997). Nevertheless, with the presence of favourable microhabitats and shelters, Chrominae are intensively predated by a wide range of carnivorous organisms ranging from resident-benthic to generalist-pelagic ones (Hixon et al. 1997).

8.3 LIFESPAN DATA

Lifespan data in the wild or even in captivity are not available for many Pomacentridae species, since these species build huge schools and have few interspecific differences making it impossible to identify them individually. Despite this, indirect evidence such as high adult mortality and very rapid growth (80% of maximum size reached within the first year) clearly indicates that these animals are short-lived in the wild and they are considered, by definition, a model for short-lived reef inhabitants (Wantiez and Thollot 2000). On the contrary, clownfishes live in small assemblages associated with a distinctive sea anemone, making their identification easy. The presence of the interspecific relationship between clownfish and anemones proved to have a significant impact on the population mortality rate that is lower than the one observed in other coral reef fishes or in the aforementioned species, of the same size, belonging to Pomacentridae (Munro and Williams 1985; Eckert 1987; Aldenhoven 1986; Buston and García 2007). Field investigations in several study sites on wild populations of clownfish demonstrated a low annual mortality rate ranging from 12.9% (Salles et al. 2015) to 13.7% (Buston 2003). In populations of *A. percula* in Madang Lagoon, Papua New Guinea, it was demonstrated that the low mortality, and consequently the predatory pressure on local populations, was not equally distributed according to the different stages of adulthood, with a mortality up to five times higher in non-breeding males (low-rank individuals) if compared to breeding couples (high-rank individuals) (Buston 2003). In the population of the same species living at Kimbe Island in Papua New Guinea, the mortality rate among the various social ranks did not produce statistical evidence although it was possible to determine that the annual mortality remained quite low compared to the one described in other reef fishes. In the case of Kimbe Island, the biannual mortality rate of local populations ranged from 18% to 49% for juveniles or immature males, from 9% to 44% in mature males, and from 19% to 55% in dominant females (Salles et al. 2015). In addition to mortality studies, an in-situ investigation performed using the recapture probability techniques demonstrated a lower bound of about 30 years in the estimated maximum lifespan of *A. percula* (Buston and García 2007). The long lifespan of clownfishes in the wild has been confirmed by a survey questionnaire (Table 8.1) distributed to researchers working with clownfishes and to public aquariums across Europe (Sahm et al. 2019).

The results of this survey demonstrated that a) for several clownfish species, the lifespan in captivity is more than a decade, with species living more than 20 years, as in the case of *A. melanopus* and *A. ocellaris*, and b) all the considered individuals in the survey were not approaching the limit of their lifespan, being actively spawning and showing no reproductive senescence (Sahm et al. 2019) (Figure 8.1).

8.4 TRANSCRIPTOMIC ANALYSIS OF ANEMONEFISH FOR LONGEVITY STUDIES

8.4.1 Amphiprioninae vs Chrominae: Positively Selected Genes

The low intrinsic mortality described earlier is correlated to anemonefishes' low predatory pressure, leading to an extraordinarily long life for all the species belonging to the subfamily Amphiprioninae. These observations are quite interesting, especially comparing the ageing of clownfishes with the short life span that characterizes the other

TABLE 8.1
Maximum Lifespan Registered by Clownfish Survey

***Amphiprion* species**	**Maximum size (cm)**	**Maximum lifespan registered**	**Status at census**
clarkii in wild	15	12	Alive
clarkii (private aquarium)	15	16	Alive
clarkii	15	9	Alive
frenatus	14	18	Dead
melanopus	12	21	Alive
ocellaris (private aquarium)	11	22	Alive
ocellaris	11	17	Alive
perideraion	10	18	Alive
akydinos	9	13	Dead

Source: as described by Sahm et al. (2019).

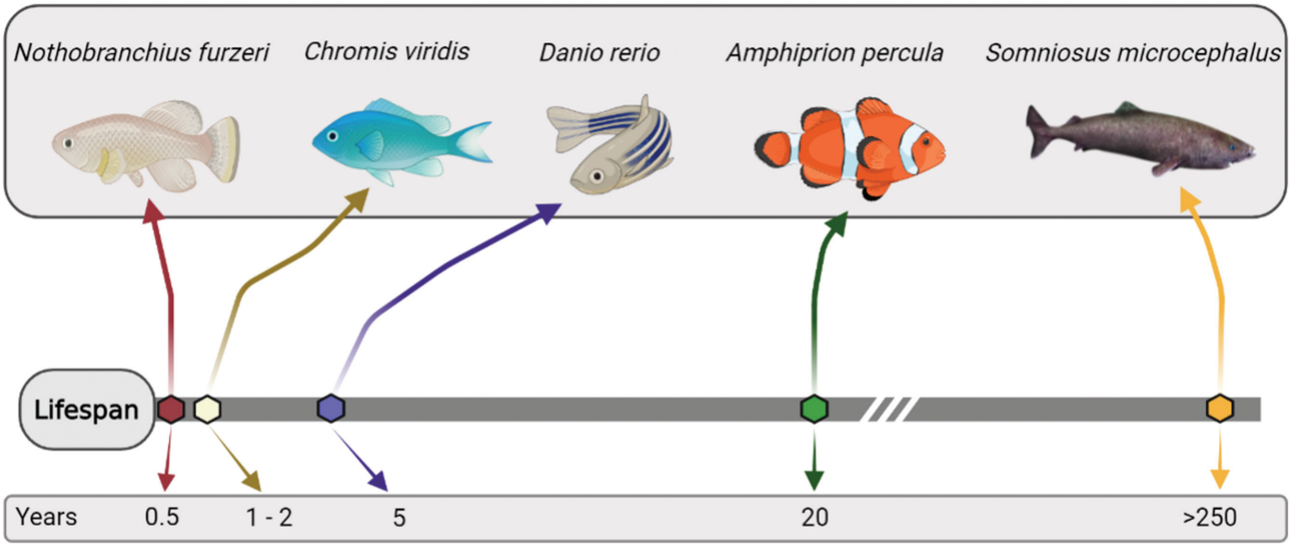

FIGURE 8.1 Lifespan in four teleost species and one elasmobranch. In established model species, such as *Nothobranchius furzeri* and *Danio rerio*, the lifespan ranges from six months to five years respectively. In the common damselfish such as blue damsel *Chromis viridis*, the lifespan in wild population could reach two years. On the contrary, clownfishes (*Amphiprion percula*) can easily reach a lifespan of 20 years and *Somniosus microcephalus* can live for more than 250 years. Created with BioRender.com.

species of the Pomacentridae family. In Amphiprioninae vs Chrominae, a total of 157 positively selected genes were identified belonging to 19 biological processes, several of them interesting for ageing research. In particular, nine of these are associated with the metabolism of xenobiotics, detoxification, and glutathione metabolism. These processes are up-regulated in experimental conditions promoting long life, such as dietary restriction, manipulation of mitochondrial translation (Houtkooper et al. 2013), or somatotropic axis inhibition, using common model organisms such as the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* (McElwee et al. 2007), various mice laboratory strains (McElwee et al. 2007; Plank et al. 2012; Amador-Noguez et al. 2007; Steinbaugh et al. 2012), rats, pigs, and rhesus monkeys (Plank et al. 2012). Clownfishes, and mole rats too, show a positive selection of two lysosomal membrane proteins LAMP2 and CD63 (LAMP3) (Sahm et al. 2019) playing an important role in chaperone-mediated autophagy, lysosomal protein degradation in response to starvation (Berditchevski and Odintsova 2007; Eskelinen 2006), and a still unknown role in adaptive immune response and apoptosis (Tanaka et al. 2020). Lysosomal dysfunction is one of the key hallmarks of ageing (Carmona-Gutierrez et al. 2016). When the function of the lysosomal pumps is impaired, it leads to an increase in lysosomal pH (Colacurcio and Nixon 2016) reducing the activity of lyases and leading to the widespread age-dependent accumulation of lysosomal aggregates (Sacramento et al. 2020) such as lipofuscin (Brunk and Terman 2002) and ubiquitin-positive inclusions (Gray et al. 2003). A marker of lysosomal dysfunction is also the conserved up-regulation across tissues and species of genes coding for proteins of lysosomal pathways that probably is an effort for a compensatory response (Aramillo Irizar et al. 2018; de Magalhães, Curado, and Church 2009; Kurz et al. 2008). Earlier findings associated selection on lysosomal genes with evolution of mammalian longevity (Li and De Magalhães 2013). Considering these results, it is reasonable to think that positive selection related to lysosomal function is one of the processes that trigger the evolution of extraordinarily long life in clownfishes. Analysis of age-dependent protein aggregation would be important to further investigate the lysosomal function in the clownfish. Other biological processes that have been observed to be under positive selection in clownfish are translation, inflammation, and autophagy. Inflammation and autophagy impairments are considered evolutionary-conserved key hallmarks of ageing (López-Otín et al. 2013) and reduction of translation rates is associated with lifespan extension in nematodes and mice (Hofmann et al. 2015; Steffen and Dillin 2016).

8.4.2 Ageing and Anti-Parallel Evolution

Transcriptomic analysis performed on various Amphiprioninae vs Chrominae (Sahm et al. 2019) and killifishes (Baumgart et al. 2016) showed signs of anti-parallel evolution, *id est* a process by which the same genetic pathways show signatures of positive selection in two lineages that evolved lifespan in opposite directions, as in the case of GSTK1, a protein involved in glutathione metabolism and protection from oxidative stress. This gene was demonstrated to be positively selected both in clownfishes and in very short-lived annual killifishes. Signs of convergence were observed in genes linked to the biogenesis of mitochondrially encoded proteins, as in the case of FASTKD2 and FASTKD5, involved in the biogenesis of mitochondrial ribosomes (Sahm et al. 2019). It is remarkable to observe that those signs of positive selection detected in both short-lived species (*Nothobranchius furzeri*) and long-lived species (clownfish and mole-rat) (Sahm et al. 2019) have been corroborated by analysing the expression of MTERF, a gene that acts as a negative regulator of mitochondrial transcription (Roberti et al. 2009). These positively selected genes involved in functions like “Mitochondrial large/small ribosomal subunit” (GO:0005762/GO:0005763) and “Mitochondrial respiratory chain complex I” (GO:0005747) have been detected in numerous species with unique lifespan, like ants (Roux et al. 2014) and African mole-rats (Sahm and Cellerino 2017; Sahm et al. 2018), as well as in Amphiprionidae (Figure 8.2). Since detailed structures of this protein are available (Ladner et al. 2004; Wang et al. 2011), homology modelling was possible, and it strongly indicates that positive selection targeted positions that are implicated in the enzymatic activity and function of the encoded protein.

Paradoxically, the short lifespan of annual killifishes of the Nothobranchiidae family, of the genus *Callopanchax* from West Africa (Cui et al. 2019) and the species *Austrofundulus limnaeus* from South America (Wagner et al. 2018) was associated with an enrichment of positively selected genes for a gene-set that stands explicitly for mitochondrial biogenesis (Sahm et al. 2017). Mitochondrial biogenesis and mitonuclear balance were related to the increase in longevity in experimental studies in several

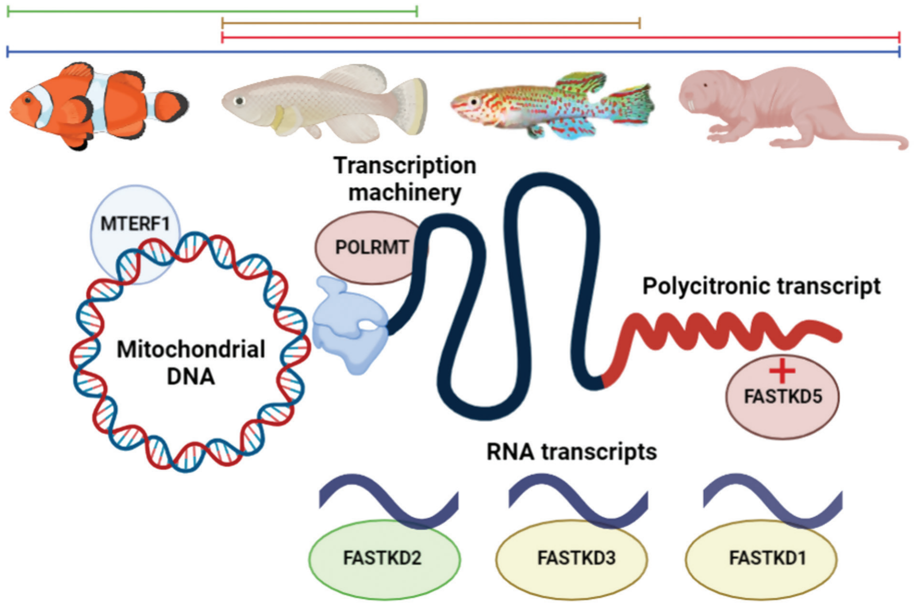

FIGURE 8.2 Convergent evolution of positively selected genes involved in mitonuclear balance in species characterized by a very short (*Nothobranchius furzeri* and *Callopanchax occidentalis*) and long lifespan (clownfishes and mole rats). In particular, the process depicted corresponds to the transcription and transcript processing of mitochondrially encoded genes. The color code of the genes corresponds to the upper bars indicates the species where the positive selection is observed: blue (MTERF1) indicates all four species, red (FASTKD5) the two killifishes and the mole rat, green (FASTKD2) *N. furzeri* and clownfishes, and brown the two killifishes. Created with BioRender.com.

model organisms (Karpac and Jasper 2013) and demonstrated to be key pathways in the regulation of ageing and lifespan (Houtkooper et al. 2013). Further investigations demonstrated that the same pathway is under positive selection in both short- and long-lived species. For example, the termination factor mTERF1 is under positive selection in clownfishes, killifishes, and mole rats (Sahm et al. 2019), and genes coding for mitochondrial ribosomal proteins and for members of complex I of the respiratory chain are under positive selection both in killifishes (Sahm et al. 2018; Cui et al. 2019; Sahm et al. 2017) and in long-lived rodents (Sahm et al. 2018) indicating that the same genetic design triggers both evolution of longevity and reduced life expectancy (Holtze et al. 2021). Therefore, some biological processes as in the case of mitochondrial biogenesis could be considered as a core genetic substrate in the evolution of lifespan. It has been probably recruited multiple times independently, in various species for various ecological adaptations, causing a modulation of lifespan in the opposite direction (short-lived vs long-lived species) depending on the life-history strategy that was selected for each evolutive clade (Holtze et al. 2021; Sahm et al. 2019).

8.5 CONCLUSION

In the 20th century, biological investigations faced a transition from descriptive to a mechanistic understanding of the biological processes leading to the conscious decision to employ model organisms as effective tools to study life. Although experimental organisms do not necessarily have to be representative of species other than themselves, in many cases model organisms should assure a wide representation of biological diversity and should allow researchers to observe phenomena that are arguably not directly observable using other target organisms, for various reasons. Since the proposal of anemonefish as model organisms suitable for ecological and, afterwards, evo-devo studies, several investigations have made use of anemonefish experimental and unique advantages. Anemonefish is an organism so convenient to study a wide range of biological phenomena that researchers are developing tools and resources specifically designed, such as collections of techniques and methods and genetic databases. For example, processes, genes, and specific sites of genome under positive selection represent potential and promising targets for follow-up studies in various scientific fields. One example of the potential application of these studies on the positively selected sites is given by the use of CRISPR/Cas9 technology in order to substitute the amino acids of a long-lived species at a positively selected site with that of a short-lived species. Considering that genomes of 12 different clownfish species are available (Pryor et al. 2020), that they can be easily cultured in captivity (Roux et al. 2021) and that several species are currently used as models to experimentally induce sex reversal (Casas et al. 2016) and pigmentation phenotypes (Salis et al. 2019), we can affirm that anemonefishes are efficient model organisms that assure a representation of biological phenomena. As a model, they represent a larger group of organisms beyond themselves and serve as the basis for articulating processes thought to be shared across several other types of organisms. For this reason, this model is powerful and gives an effective repertoire of answers to scientific questions in modern investigation not only related to ageing theories but also considering other research fields. Anemonefish are no doubt the first long-living experimental fish model for ageing studies, but they represent a fundamental model for many scientific fields due to their biological, physiological, and genetic peculiarities.

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