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Shaping the cardiac response to hypoxia: NO and its partners in teleost fish

Sandra Imbrogno [a,1], Tiziano Verri [b,1], Mariacristina Filice [a], Amilcare Barca [b], Roberta Schiavone [b], Alfonsina Gattuso [a], Maria Carmela Cerra [a,*]

[a] Dept. of Biology Ecology and Earth Sciences, University of Calabria, Arcavacata di Rende, Italy
[b] Dept. of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy

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ABSTRACT

The reduced availability of dissolved oxygen is a common stressor in aquatic habitats that affects the ability of the heart to ensure tissue oxygen supply. Among key signalling molecules activated during cardiac hypoxic stress, nitric oxide (NO) has emerged as a central player involved in the related adaptive responses. Here, we outline the role of the nitrergic control in modulating tolerance and adaptation of teleost heart to hypoxia, as well as major molecular players that participate in the complex NO network. The purpose is to provide a framework in which to depict how the heart deals with limitations in oxygen supply. In this perspective, defining the relational interplay between the multiple (sets of) proteins that, due to the gene duplication events that occurred during the teleost fish evolutive radiation, do operate in parallel with similar functions in the (different) heart (districts) and other body districts under low levels of oxygen supply, represents a next goal of the comparative research in teleost fish cardiac physiology.

1. Introduction

The ability to cope with environmental stress is fundamental for the homeostatic response of living organisms. Following Selye's first studies on the stress response (Selye, 1978), and the definition of *"distress"* and *"eustress"* (Selye, 1976), decades of research attempted to describe the interplay between the environment and the organism, the mechanisms of physiological adaptation, and the limits of stress tolerability. Results, far from being definitive, highlight an intricate network of stimuli and responses that, recently, have been tentatively recapitulated in the concepts of *stressotope* (the multilevel adaptive contest from the molecular circuits, up to the population level) and *stressome* (changes in genes, proteins and metabolism in the presence of maladaptive stress) (Balasch and Tort, 2019).

Organisms physiologically respond to environmental stress with two major strategies either by preserving a normal performance, also in terms of growth and reproduction (capacity adaptation), or by enhancing resistance at the expenses of homeostasis, while maintaining the ability to recover when conditions return favourable (resistance adaptation) (Cossins and Bowler, 1987). These strategies are differently expressed by the individuals of a given species due to the different phenotypic plasticity and fitness (Sloan Wilson et al., 1994).

In water environments, animal populations are continuously challenged by fluctuations in abiotic and biotic parameters, including O_2, temperature, pH, salinity, organic compounds, and nutrient availability. These are natural events that organisms face by activating a large variety of physiological strategies which characterize a given species and are the result of a long adaptive history. An example is the extraordinary ability of cyprinids to cope with reduced O_2 availability, even tolerating anoxia. By involving genes and proteins recruited in energy turnover (Hochachka and Somero, 2002), under low O_2 these teleosts decrease bioenergetic demand/production balance with the subsequent preservation of energy, osmotic equilibrium, and substrates (Imbrogno et al., 2019a).

Adaptation to environmental challenges requires that crucial organs maintain their performance, by activating strategies of preservation. Among them, the heart represents a key player in the physiological plasticity of fishes in response to environmental stress (Gamperl and Farrell, 2004; Jayasundara and Somero, 2013; Nyboer and Chapman, 2018). For example, according to the "oxygen- and capacity-limited thermal tolerance" (OCLTT) concept, changes in temperature, systemic oxygen demand and delivery (Portner and Farrell, 2008) may play important roles in setting thermal limits of organisms (Pörtner et al., 2017; Portner and Knust, 2007). In this scenario, adaptive plasticity of

* Corresponding author.
E-mail address: maria_carmela.cerra@unical.it (M.C. Cerra).
[1] These authors equally contributed to the work.

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cardiac performance may be critical to compensate for the increased oxygen demand (Anttila et al., 2013; Pörtner et al., 2017). This means that the capacity of the heart to guarantee a continuous blood supply to all metabolic active tissue represents the primary determinant of stress tolerance, since it defines the upper limit of the adaptive response. This is particularly important in the present historical moment in which the environment is endangered by an increasing number of perturbations, also due to uncontrolled human manipulation, so that the upper limits of adaptations are severely threatened.

By using teleosts as a frame of reference, and after a brief outline on major aspects of hypoxia in aquatic environments and on fish oxygen sensing ability, we will provide an updated insight on the cardiac strategies in the fish adaptive response to hypoxia, with focus on the role of the adrenergic and the NOS/NO system.

We will also attempt to highlight the complexity encountered in teleost fish molecular systems in which, frequently, similar proteins exert similar functions. In many cases, this is the result of gene duplication events, occurred during teleost fish evolutive radiation, and that led to some very complex genomic patterns, as in the case of Cyprinids and Salmonids [see e.g. (Glasauer and Neuhauss, 2014; Omori and Kon, 2019)]. With this in mind, each teleost species should be considered as a biological singularity to fully understand the physiological bases of the adaptation to the various environments and the environmental conditions each species may experience.

2. Aquatic hypoxia

Hypoxia is a condition of low oxygen common to aquatic environments with low mixing, light limitations, and/or high organic decomposition (Chapman, 2015). Aquatic organisms routinely experience hypoxia under a diurnal or a seasonal basis, and this importantly challenges animal performance. Fish differently respond to O_2 limitations, moving from highly intolerant species to species that tolerate hypoxia and even anoxia. For example, salmon and tuna strictly rely on aerobic metabolism for fast swimming, while species including eel, carp and hagfish are capable of maintaining a proper performance under hypoxia/anoxia (Gattuso et al., 2018). The formidable hypoxia tolerance of several species is exemplified by the classic experiment on the Indian cyprinid teleost *Rasbora daniconius* which is able to survive up to 102 days in a hermetically sealed glass jar (Mathur, 1967). The high variability of fish species to inhabit hypoxic, and even anoxic, environments precludes a general hypoxia threshold for aquatic environments. Some studies proposed the onset of hypoxia at $\leq$2.0 mg O_2/l (Diaz and Rosenberg, 2008), or 2.8 mg O_2/l (Mu et al., 2017; Wu, 2002), or 3.0 mg O_2/l (Howell and Simpson, 1994), or 4.0 mg O_2/l (Paerl et al., 2006). Others defined hypoxia referring to O_2 levels that negatively impact a target species (Pollock et al., 2007). Accordingly, hypoxia appears to be context-dependent and requires the integrated evaluation of organism tolerance, abiotic and biotic traits of the environment, and duration of exposure (Chapman, 2015; Chu and Tunnicliffe, 2015; Hrycik et al., 2017).

Fish show the ability to "sense" O_2 variations in water and blood thanks to the presence of neuroepithelial cells (NECs) (Jonz et al., 2016) located on gill arches, on the lungs of adult water- and air-breathing fish (Jonz et al., 2004, 2016; Porteus et al., 2012; Zaccone et al., 1992, 1997), on the skin (Coccimiglio and Jonz, 2012), and on the epithelia of accessory respiratory organs [see for example some catfish species (Zaccone et al., 2018)].

NECs differently "sense" hypoxia and this correlates with the degree of animal tolerance. For example, in hypoxia tolerant fish, as in the case of the Cyprinid teleost, the goldfish *Carassius auratus*, NECs are activated only when PO_2 decreases to deep hypoxia or even anoxia and this is accompanied by an increased ventilatory response (Tzaneva et al., 2011).

The molecular and cellular mechanisms allowing O_2 sensing have been largely explored in mammals, and only partially in fish. In both it was found that they require a modulation of O_2 sensitive K^+ channels, and thus of K^+ current, activation of voltage-gated Ca^{2+} channels, and Ca^{2+} influx. The consequence is the release of one or more transmitters with final effects on ventilation/perfusion [for references and review: (Dzal et al., 2015; López-Barneo et al., 2016; Prabhakar and Peers, 2014)].

Changes in mitochondrial function and ATP formation are the obvious consequences of O_2 fluctuations. In fish, still limited evidence suggests that mitochondria contribute to O_2 sensing. Data in the hypoxia tolerant crucian carp indicate a role for AMP-dependent kinase (AMPK), with a consequent metabolic depression in heart and brain only under anoxia (Stenslokken et al., 2008). Accordingly, AMPK and its downstream effectors, may be switched on only when it becomes strictly necessary, thus enhancing animal resistance to low O_2 (Pamenter, 2014).

Robust data indicate that in fish, nitric oxide (NO), carbon monoxide (CO), and hydrogen sulphide (H_2S), are involved in O_2 chemoreception. This occurs via an upstream modulation of gasotransmitter generation, and via a downstream control of ion channels activity (e.g. those mediating K^+ and Ca^{2+} currents). For detailed information on these mechanisms see (Gattuso et al., 2018; Prabhakar and Peers, 2014; Tzaneva and Perry, 2014, 2016).

3. Cardiac adaptive responses to O_2 limitation

A proper cardiac dynamic is fundamental for the adaptive response to O_2 fluctuations (Filice et al., 2021b; Gamperl and Driedzic, 2009; Stecyk, 2017; Tota et al., 2011). At the same time, the heart requires an appropriate O_2 supply to sustain an adequate performance. Accordingly, a virtuous circuit is necessary to maintain the activity of the O_2-consuming working heart when O_2 is limited, and the periphery requires to be oxygenated.

3.1. Physiological strategies

The physiological response of the fish heart to O_2 availability is the result of a long-term adaptation in which a number of orchestrated events, from molecular to cell and tissue level, converge to shape the whole organ performance to the restricted availability of the gas, also limiting the potential damages for cells and tissues.

In mammals, heart oxygenation is provided by a rich coronary vasculature that perfuses the compact myocardial muscle. Differently, fish possess a "venous heart" that receives deoxygenated blood from the body into a *sinus venosus*. The blood moves into a single atrium, a single ventricle and lastly into the *bulbus arteriosus* to reach the gills for gas exchange. O_2 delivery to the myocardium depends on the species-specific architecture of the cardiac wall. In some species, the whole ventricular wall is composed of an avascular spongy myocardium (*spongiosa*) supplied by diffusion from the ventricular cavity. Different from this organization, the inner spongy musculature may be outlined by an outer compact layer (*compacta*) served by coronary arteries arriving from the respiratory regions, thus delivering oxygenated blood [for extensive review on the different types of myocardial organization in fish see: (Farrell and Jones, 1992; Icardo et al., 2005; Tota et al., 1983)]. As a consequence, the inner spongy region of the fish heart is constantly exposed to low O_2. At the same time, also the compact layer experiences a drop in O_2 partial pressure under hypoxia. This is critical for the heart which continues to work also when O_2 supply is reduced.

Changes in O_2 availability may differently influence the relative performance of the two myocardial layers. This is supported by data in salmonids that show a different behaviour of the ventricular strips from *compacta* vs *spongiosa* in terms of network, shortening and lengthening when exposed to low O_2 [for details see: (Roberts et al., 2021; Roberts and Syme, 2018)]. Interestingly, these effects disappear when O_2 is restored, showing that they do not represent a myocardial failure but an adaptive, protective trait of the heart (Roberts et al., 2021).

A general physiological response of the fish heart to hypoxia is bradycardia, accompanied by a depression of myocardial contractility and O_2 consumption rates. This provides a strategy that, by depressing ATP requirement, reduces the need of O_2, thus protecting the heart (Farrell, 2007). This response is particularly evident in hypoxia-intolerant species (Agnisola et al., 1999; Carnevale et al., 2020). In contrast, a normal or even enhanced cardiac function is present in species showing hypoxia/anoxia tolerance (Imbrogno et al., 2014, 2019a; Stecyk et al., 2004), as in the case of Cyprinids (Bickler and Buck, 2007). However, in Cyprinids, the cardiac response to O_2 fluctuations is characterized by remarkable interspecific differences. For example, in the common carp (*Cyprinus carpio*), a decreased heart function is observed after 24 h of severe hypoxia. This is associated with a reduced requirement of ATP and O_2 for the tissue (Farrell, 2007). Contrarily, the crucian carp (*Carassius carassius*) preserves a normal cardiac activity and autonomic cardiovascular control up to five days of anoxia. This is attributed to a low routine ATP demand of the cardiac tissue, and a sufficient anaerobic generation of ATP that sustains the heart performance. Thus, bradycardia is not necessary for energy preservation (Stecyk et al., 2004). In addition, the goldfish *C. auratus*, able to survive long periods under hypoxia, even tolerating anoxia, shows a hypoxia-dependent potentiation of cardiac performance (Filice et al., 2021a). This effect, observed on the isolated and *in vitro* perfused whole heart, deprived of the nervous and humoral control, is mainly expressed by an increased intrinsic myocardial sensitivity to the Frank-Starling mechanism (Imbrogno et al., 2014), that represents a major regulator of the cardiac performance in teleosts (Amelio et al., 2013; Olson, 1998; Shiels and White, 2008).

The experience of low O_2 in the venous heart of fish is accompanied by an intrinsic preconditioning-like behaviour. In mammals, preconditioning is a manoeuvre that takes advantage by the ability of short periods of stress (e.g. hypoxia, ischemia, stretch, heat shock), or administration of specific drugs, to induce myocardial resistance and prevent the damages induced by ischemia/reperfusion (Rocca et al., 2019, 2020). The same phenomenon appears to exist in fish and characterizes not only the compact layer but also the spongy myocardium, which is normally exposed to low O_2 partial pressure (Gamperl and Farrell, 2004). This is revealed by data on the zebrafish (*Danio rerio*) showing that samples first exposed to non-lethal hypoxia (PO_2 = 15 mmHg) and then to severe hypoxia (PO_2 = 8 mmHg), are characterized by an increased survival time (Rees et al., 2001). Also, in the rainbow trout, a brief pre-exposure (5 min) of the *in situ* heart to low PO_2 (5–10 mmHg) fully abolishes the severe detrimental effects on the cardiac performance elicited by a long exposure (15 min) to anoxia (Gamperl et al., 2001).

3.1.1. The role of the adrenergic system

For many fish species, an important trait of the cardiac response to hypoxic stress is the involvement of the adrenergic system (Farrell et al., 1986). After activation of peripheral O_2 chemosensors, the adrenergic outflow is positively modulated at the level of both nerve terminal and chromaffin release (Farrell and Jones, 1992; Nilsson and Holmgren, 1992). Despite there is debate about the role of humoral *vs* neuronal catecholamines (CAs) on the respiratory and cardiovascular modulation [see for references (Pan and Perry, 2020)], adrenergic activity adjusts tissue blood perfusion so that O_2 demand is balanced by an adequate supply (Buckler, 2007; Milsom and Burleson, 2007). The fish heart itself is able to release CAs under low O_2. One example is the goldfish, whose heart contains intracardiac chromaffin tissue (Cameron and O'Connor, 1979) and a rich adrenergic innervation (Imbrogno et al., 2019b; Newton et al., 2014). Another example is the hypoxia-tolerant tropical *Pygocentrus nattereri* (red-bellied piranha), in which the heart contains CAs (adrenaline: 7.27 ng/g; noradrenaline: 14.48 ng/g) in an amount comparable to that detected in the carp [adrenaline: 3.2 ng/g; noradrenaline: 16.2 ng/g; (Temma et al., 1989)], and lower than that reported in cod [adrenaline: 170 ng/g; (v. Euler and Fänge, 1961)]. The release of CAs from the heart of the red-bellied piranha allows to restore force development, which is almost halved after long exposure to limited O_2 (Joyce et al., 2019). The effect is possibly obtained by increasing intracellular Ca^{2+} via L-type Ca^{2+} channels and sarcoplasmic Ca^{2+} release (Joyce et al., 2019).

The beneficial effect of CAs on the fish heart exposed to hypoxia is confirmed by classic and novel observations (Farrell and Smith, 2017; Gesser et al., 1982; Hanson et al., 2006; Stecyk et al., 2011). In the cod (*G. morhua*), low O_2 rapidly decreases the ventricular work if it is not sustained by epinephrine (Syme et al., 2013). In addition, high adrenergic stimulation protects the myocardium of rainbow trout from the hypoxia-dependent acidosis and hyperkalemia, also preventing heart collapse (Hanson et al., 2006). Despite the general consensus about the influence of CAs on the hypoxic fish heart, species-specific differences prevent a univocal picture. For example, stress-tolerant fish, such as the members of the genus *Anguilla*, do not show increased plasma CA in response to stressful stimuli (Imbrogno, 2013). Also in the pacu (*Piaractus mesopotamicus*), a significant adrenergic response during hypoxia is absent (Perry et al., 2004). In contrast, in two Amazonian teleosts (traíra, *Hoplias malabaricus*, and jeju, *Hoplerythrinus unitaeniatus*), CAs are released during very severe reduction of O_2 availability (Perry et al., 2004).

As in mammals, in fish, CAs may target the heart thanks to the presence of adrenergic receptors (ARs). It was initially believed that teleost cardiac regulation mainly occurred via β2-ARs. However, it is now recognized that also β3-ARs are expressed in the teleost heart (Nickerson et al., 2003; Petersen et al., 2013). Accordingly, the activation of different receptor subtypes may result in different and sometimes opposite effects on the heart (Imbrogno et al., 2015a). In addition, in fish, β-ARs are differently expressed by the myocardial regions. This reinforces the morpho-functional heterogeneity of the fish heart, described above in this review, in terms of architectural traits and myocardial O_2 supply. It was found that the spongy and the compact myocardial layers differently express ARs. In chinook salmon (*Oncorhynchus tshawytscha*), the *spongiosa* shows a 14% higher density of β-ARs with respect to the *compacta* (Gamperl et al., 1998). Consequently, they are differently sensitive to CAs. This may be important for maintaining the function of the two myocardial layers, regardless of the different O_2 availability that they experience, particularly during hypoxia. Recent evidence shows that cardiac β3-ARs are important mediators of the CAs-dependent response to hypoxia (Imbrogno et al., 2006, 2015a). These receptors are involved in the basal adrenergic cardiovascular regulation of both adult and larval fish. Data from the rainbow trout (Petersen et al., 2013) and the adult eel heart (Imbrogno et al., 2006) exposed to normoxia, show that β3-AR stimulation depressed myocardial dynamics and increased heart rate, also involving a compensatory baroreflex mechanism (Petersen et al., 2013). β3-ARs are also recruited when the teleost heart is exposed to hypoxia. However, their role seems to differ in tolerant *vs* intolerant species. In the O_2-sensitive trout (*O. mykiss*), a reduced expression of cardiac β3-AR accompanies the decrement in cardiac pumping capacity after long acclimation (17–23 weeks) to moderate hypoxia (~40% air saturation) (Motyka et al., 2017). Differently, in the hypoxia-tolerant goldfish, the time-dependent increase of contractility, that characterizes the response of the heart to hypoxic stress, recruits functional cardiac β3-ARs, acting via a cAMP-dependent signalling (Leo et al., 2019).

Of relevance for the fish heart under hypoxia is the protection elicited by adrenergic stimulation against acidosis. This was classically observed in both perfused hearts (Farrell et al., 1983, 1986; Farrell and Milligan, 1986) and isolated cardiac strips (Gesser et al., 1982; Gesser and Jorgensen, 1982), in which the acidosis-dependent impairment of the myocardial performance is rescued by adrenaline administration (Farrell et al., 1983).

3.2. Metabolic strategies

Under limited O_2, an appropriate cardiac function and blood delivery allow to mobilize huge amounts of glucose from the hepatic glycogen stores, and to supply with fuel substrates tissues and organs. In parallel, metabolism is depressed, thus preserving glycogen stores, and oxidative phosphorylation is downregulated (Bickler and Buck, 2007; Vornanen and Haverinen, 2016). Both events ensure heart endurance when O_2 deprivation occurs for a long time. At the same time, there is the need to escape from acidosis caused by increased anaerobic waste-products [references in (Gattuso et al., 2018)]. Cyprinids, such as the goldfish and the crucian carp, are able to reduce lactate to ethanol, a harmless anaerobic end product, that can be easily excreted through the gills (Dhillon et al., 2018; Shoubridge and Hochachka, 1980). Lactate reduction mainly occurs in the swimming muscle, which not only generates the ion because of the intense activity, but also receives that produced by other regions such as brain, liver and heart (Fagernes et al., 2017; Torres et al., 2012). In the goldfish, the heart itself may prevent anaerobic lactate accumulation via a modulation of the fructose-1, 6-bisphosphate aldolase B, a specific hypoxia-regulated protein (Imbrogno et al., 2019a). The result is a balance between glycolytic vs gluconeogenic fluxes that allows the recycling of pyruvate, the first product of anaerobic glycolysis (Imbrogno et al., 2019a). At the same time, this may mitigate the negative consequences of a possible low hypoxia-dependent ATP production. This molecular shift is important since it contributes to protecting the heart from acidosis that, as largely shown in mammals, severely impairs myocardial contractility/relaxation and reduces the pumping ability of the heart (Orchard and Cingolani, 1994; Orchard and Kentish, 1990).

A relevant aspect in the metabolic adaptation of the teleost heart to hypoxia is the contribution of the different myocardial regions. In fact, as shown in the bluefin tuna (*Thunnus thynnus*), the inner ventricular *spongiosa* has a more efficient lactate metabolism than the compact outer myocardium. This parallels the different degree of oxygenation experienced by the two myocardial regions (see above), allowing the *spongiosa* to cope with a limited O_2 supply [for review see (Tota et al., 2011)].

3.3. A gene perspective

The physiological evidence outlined above, often controversial if different tissues, organs and/or species are considered, find a justification in the complex pattern of gene products that emerge when all the various protein sequences are studied at the same time and in the same framework, as highlighted after deep comparative genomics analyses. Fig. 1 depicts the complexity of the ARs (i.e. ADRB1, ADRB2 and ADRB3 type proteins) array from a number of teleost species, selected on the basis of their position in the teleost fish tree of life and their experimental use. All of the teleost fish reported are Otophysi, with the exception of the European eel, which is an ancient teleost fish, the spotted gar, which lineage diverged from teleosts before teleost genome duplication (thus bridging teleosts to tetrapods), and the mouse, which is included as a mammalian reference species. It is conveivable that, as

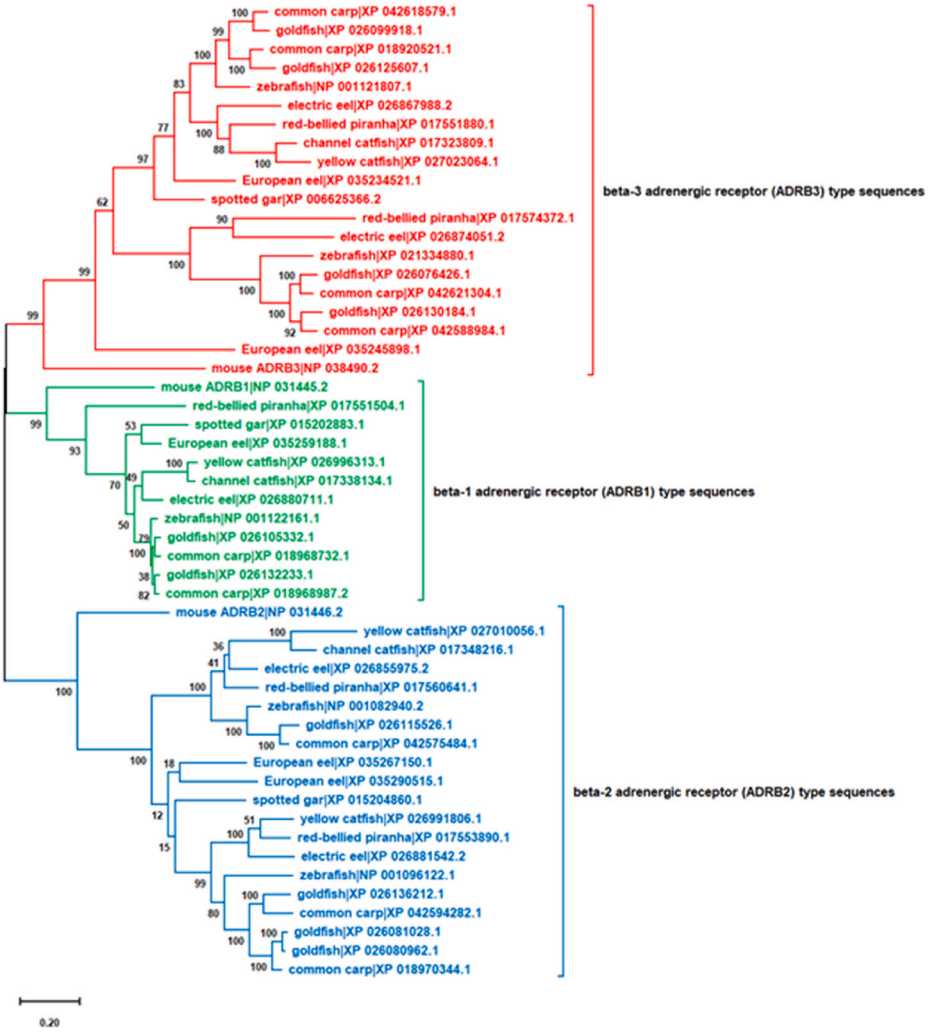

Fig. 1. The evolutionary history of adrenergic receptors was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−23184.46) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 52 amino acid sequences. There were a total of 616 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

soon as the number of duplicated genes rises, the number of receptor proteins that can putatively perform similar functions increases. As expected, the species that exhibit maximal complexity are the goldfish and the common carp, among Cyprinids, although proteins resulting from gene duplication products are variably scattered in the evolutionary history of these organisms, as in the European eel, another teleost robustly adapted to hypoxia. This trend in increasing the number of adrenergic receptor sequences (available in databanks) is evident passing e.g. from mouse (3), to spotted gar (3), European eel (5), zebrafish (5), common carp (9) and goldfish (10) (see Fig. 1 and **Supplemental File 1** for details). This parallels the increase in the number of sequences in the ADRB2 and ADRB3 groups with respect to the ADRB1.

4. The NOS/NO system as a major player in the teleost cardiac response to hypoxia

Common sets of molecular networks and intracellular cascades are recruited in the heart of teleost fish to allow tolerance to low O_2 availability in the environment or in internal tissues. NO and its metabolites nitrite and nitrate are largely recognized for their crucial importance as molecular actors of the hypoxia-dependent physiological responses of the heart (Fago and Jensen, 2015).

Generated in mammals by three NOS isoenzymes [i.e. the constitutive endothelial (eNOS *alias* NOS3) and neuronal (nNOS *alias* Nos1), and the inducible (iNOS *alias* NOS2), isoforms], NO plays pleiotropic actions. In fish, it is fundamental for modulating the basal heart performance, and is involved in a huge variety of adaptive responses to stress (Amelio et al., 2013; Filice et al., 2017; Imbrogno et al., 2006, 2010, 2013, 2014; Tota et al., 2004).

The presence of functional NOS enzymes is largely documented in fish, although a conclusive picture is still missing. Results from physio-pharmacological, NADPH-diaphorase and immunolocalization analyses have detected the presence of an "eNOS-like" activity in the heart of several species (Amelio et al., 2006, 2008; Imbrogno et al., 2011; Imbrogno et al., 2017a,b). However, a gene for a canonical eNOS has not been identified yet. This poses the question on how and when the different NOS isoforms have evolved and suggests that in teleosts, a (set of) nNOS isoform(s) showing an endothelial-like consensus may cover some functional features of the eNOS isoform identity (Andreakis et al., 2010). An example of such protein complexity in teleost fish is shown in Fig. 2. As for ARs above, there is a trend to increase the number of NOS type proteins in Cyprinids, particularly common carp and goldfish, but also in other teleosts, such as catfishes and piranhas. Moreover, while NOS3-type proteins are absent in teleost fish, NOS1-type proteins appear more closely related to NOS3 than to NOS2 proteins. The number of nitric oxide synthase sequences (available in databanks) increases passing e.g. from mouse (3), spotted gar (3), catfishes (3), piranha (3) and zebrafish (3) to common carp and goldfish (5) (see Fig. 2 and **Supplemental File 2** for details), mainly due to duplication events involving NOS2 and NOS3 type genes. In this respect, the possibility that one of the different forms may have evolved to support eNOS-like functions cannot be *a priori* excluded.

Of remarkable importance in relation to the response to hypoxia is the mandatory requirement of O_2 for NOS enzymes for converting L-arginine in L-citrulline and NO (Moncada and Higgs, 1993). In the presence of a reduced O_2 availability, when this reaction is compromised, an alternative pathway for NO generation is represented by the reduction of nitrite. This may occur via acidic disproportionation (Zweier et al., 1999), or enzymatic reduction via xanthine oxidoreductase, mitochondrial enzymes, or deoxygenated Hb, Mb, cytoglobin-1, neuroglobin, globin-X, and eNOS [see references in: (Angelone et al., 2012; Corti et al., 2016; Giordano et al., 2020; Omar and Webb, 2014)]. Thus, under limited O_2, nitrite may function as a reservoir of NO (Lundberg et al., 2008; van Faassen et al., 2009). Also, nitrate contributes to NO homeostasis since it can be slowly reduced to nitrite by xanthine oxidoreductase (Hansen et al., 2016b; Jansson et al., 2008). Although these pathways work in parallel, under hypoxic conditions, the production of NO from nitrite seems to be more pronounced.

Under reduced O_2, the heart of hypoxia-resistant fish, such as the goldfish and the crucian carp, is able to maintain high internal nitrite levels, thus ensuring a constant source of NO availability. This occurs through an extracellular uptake of nitrite in tissues with high myoglobin (Mb) and mitochondria content, including the heart (Hansen et al., 2016a; Sandvik et al., 2012). This nitrite inward diffusion is allowed by

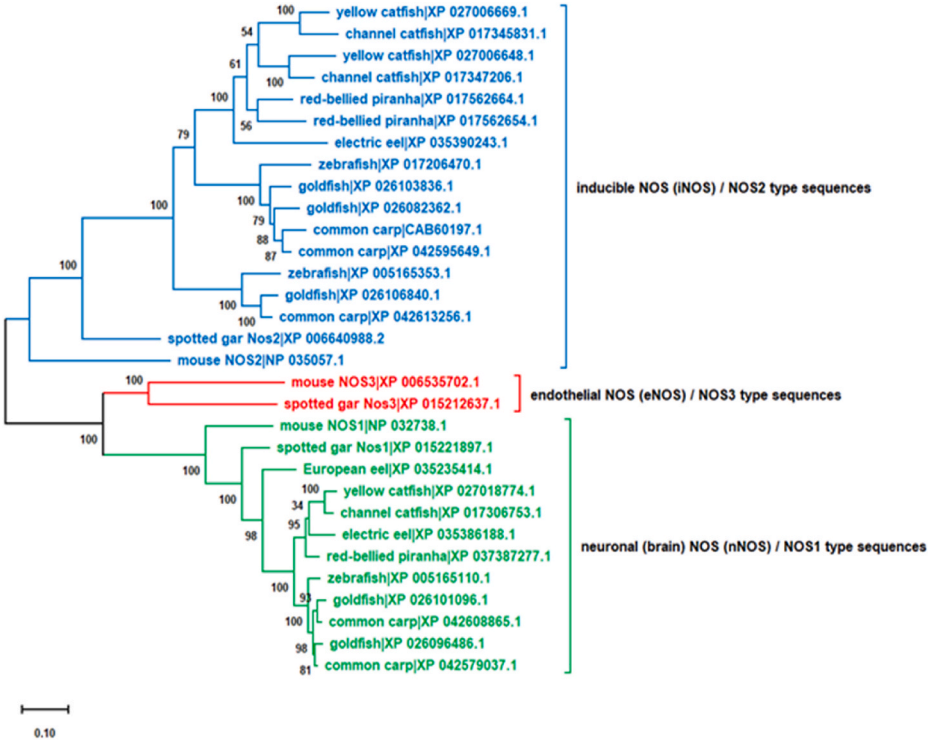

Fig. 2. The evolutionary history of nitric oxide synthases was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−29100.22) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 31 amino acid sequences. There were a total of 1531 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021). Test of phylogeny: Bootstrap method; No. of Bootstrap Replications: 100. GenBank Acc. Nos. are indicated.

proteins that keep low the cytosolic concentration of free nitrite (Hansen and Jensen, 2010).

In fish, an established effect of NO on the hypoxic heart is the competitive binding to CytC oxidase and the consequent inhibition of mitochondrial O_2 consumption (Erusalimsky and Moncada, 2007). This effect, common to hypoxia-intolerant and hypoxia-tolerant fish, enhances the force generated per O_2 consumed, and this contributes to preserving myocardial efficiency (Misfeldt et al., 2009; Shen et al., 2001). In line with this, Pedersen and coworkers (2010) observed that in the goldfish NO inhibits mitochondrial respiration without changing contractility and this sustains myocardial function in the presence of reduced oxygen. However, differences in the myocardial effects of NO on the fish hypoxic myocardium have been observed in relation to the NO source. It was found, by using ventricular strips from trout and goldfish, that the NOS-derived NO inhibits respiration rate, and improves myocardial efficiency in both species (Pedersen et al., 2010). Nevertheless, the NO generated from nitrite conversion reduces O_2 consumption without changing force development in trout but not in goldfish. This was attributed to species-specific differences in O_2 affinity of cardiac Mb and then, in its nitrite reductase capacity. In the presence of a reduced O_2 availability, trout Mb may readily de-oxygenate and generate NO from nitrite, while in the goldfish, Mb remains saturated with O_2, and this prevents nitrite reduction (Pedersen et al., 2010).

The molecular mechanisms that sustain the nitrergic modulation of the fish heart under hypoxia are still a field for exploration. In the goldfish, the potentiated heart function observed under hypoxia is accompanied by an increased expression of NOS and a consequent higher generation of NO (Imbrogno et al., 2014). The possibility that this enhanced NO supply contributes to the hypoxia-dependent increase of contractility is supported by the reduction of these effects observed when NO is removed by PTIO scavenging, and when NOS is inhibited by L-NMMA. Moreover, in the goldfish the exposure to a limited O_2 availability is accompanied by activation of PI3–K/Akt signalling (Filice et al., 2020), resembling the molecular pattern that in mammals controls the activity of eNOS, and thus NO generation (Angelone et al., 2015; Strijdom et al., 2009). This is interesting not only in an evolutionary perspective, since it appears to be a conserved mechanism for NOS regulation, but also in relation to the characterization of the NOS enzyme expressed in the fish heart that clearly maintains a functional regulatory trait of the mammalian eNOS isoform (Imbrogno and Cerra, 2017; Imbrogno et al., 2018).

Once NO is generated, it acts by regulating a large number of intracellular effectors. It positively modulates soluble guanylate cyclase (sGC) and this affects the intracellular availability of cGMP. Other NO downstream events, that modulate protein function, are the NO-dependent protein S-nitrosylation, occurring via the direct attachment of NO to a metal centre to form a metal nitrosyl complex (Heinrich et al., 2013; Hess et al., 2005), and nitration that consists in the addition of a nitro group (-NO_2) in the tyrosine aromatic ring, a process mediated usually -although not exclusively-by peroxynitrite, another NO metabolite (Halliwell, 1997; Radi, 2004). Interestingly, in the goldfish exposed to hypoxia, the intracellular mechanisms activated by NO generation are not accompanied by cGMP production (Filice et al., 2020). In addition, protein S-nitrosylation is reduced while the expression of Nox2, the catalytic subunit of NADPH oxidase (Filice et al., 2020), and of 3-nitrotyrosine (Mazza et al., 2019) increases. This opens the possibility that NO may utilize nitration for modulating the potentiated response of the goldfish heart to hypoxia. Amongst the possible proteins targeted by nitration, those controlling the molecular machinery responsible for the myocardiocytes contractility are of interest to understand the events occurring in the goldfish heart under hypoxia. An example is the SERCA2a pump. The protein is expressed in the fish heart (Imbrogno et al., 2015b; Korajoki and Vornanen, 2012) and actively transports Ca^{2+} into the sarcoplasmic reticulum, thus directly controlling the rate of contractility and relaxation. The presence of cysteine and tyrosine residues makes it susceptible to nitrosative modifications (Bigelow, 2009; Braun et al., 2019). For this reason, nitrated SERCA2a is used as a cardiac marker of nitrative stress (Bigelow, 2009). In line with the possible involvement of SERCA2a in the nitrergic-dependent control of the cardiac response of the goldfish to low O_2, the inhibition of the pump is accompanied by a significant reduction of the hypoxia-induced increase of heart performance (Filice et al., 2020). Another possible target for NO in the hypoxic fish heart is represented by sarcolemmal K_{ATP} channels that in the goldfish are positively modulated by NO, and this is supposed to increase tolerance to limited O_2 (Cameron et al., 2003). This is interesting in terms of cardiac protection since, according to data in mammals, when the myocardium is exposed to O_2 deprivation, K_{ATP} channels represent a key point of the ischemic preconditioning protective mechanism (Noma, 1983).

An aspect that deserves further consideration to better describe the role of NO in the teleost cardiac response to hypoxia is the crosstalk with other crucial molecular mediators. An example is the major hypoxia-related protein, HIF-1a [for a recent review see e.g. (Mandic et al., 2021)]. In several species, such as the Atlantic croaker [*Micropogonias undulatus*; (Rahman and Thomas, 2007)] and the Antarctic red-blooded teleost *Notothenia coriiceps* (O'Brien et al., 2020), the protein is expressed in the heart and is positively modulated by hypoxia, the effects being reversed by restoration of normoxia. Also the goldfish heart expresses HIF-1a, which can be found among an array of gene products

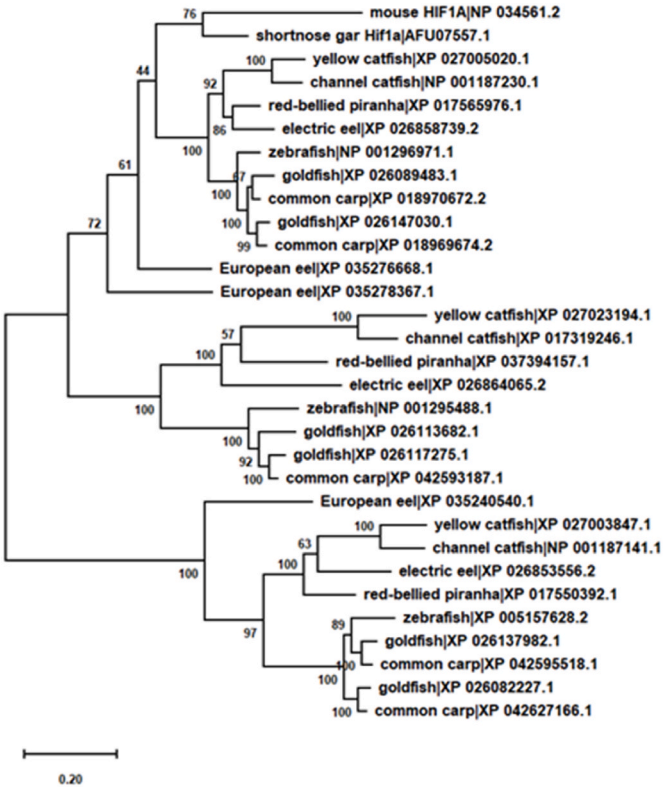

Fig. 3. The evolutionary history of hypoxia-inducible factors 1α was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−20259.29) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 31 amino acid sequences. There were a total of 914 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021). Test of phylogeny: Bootstrap method; No. of Bootstrap Replications: 100. GenBank Acc. Nos. are indicated.

(Fig. 3). Protein expression increases in the presence of O_2 limitation and this is paralleled by the enhanced expression of NOS enzyme (Imbrogno et al., 2014). This is noticeable since it resembles the events occurring in the mammalian myocardium in which, under the hypoxic stress induced by ischemia, HIF-1a contributes to cell survival by activating NOS gene expression (Jugdutt, 2002), together with other hypoxia-related genes (Hochachka and Lutz, 2001; Liu and Simon, 2004; Semenza, 2007). At the same time, NO at high concentrations (>1 μM) stabilizes HIF-1α that dimerizes and binds HIF responsive elements, thus promoting NOS expression (Mateo et al., 2003). Further studies will better clarify the interplay between HIF-1a and NOS in the fish myocardium. However, based on the available knowledge, it appears that the relationship between these important mediators is a crucial functional pathway of the hypoxia response that appeared early in the evolution and is retained up to the mammalian heart.

To fully assess the complexity of potential interactions among genes/gene products, it is worth also focusing on the number of HIF1A type proteins, as they operate in different species and/or different tissues. Similar to what described above for adrenergic receptors and nitric oxide synthases, the set of HIF1A type proteins may consistently differ in different fish species (Fig. 3). In particular, while one single HIF1A protein sequence is available in databanks from mouse and shortnose gar, this number significantly increases passing to teleost fish, such as European eel (3), catfishes (3), piranha (3), electric eel (3), zebrafish (3), goldfish (5) and common carp (6) (see Fig. 3 and **Supplemental File 3** for details).

5. Conclusions

Despite the rich information provided by decades of functional, structural, and biomolecular investigations, scientists are still fascinated by the impressive flexibility shown by the heart of several teleost species to face the challenge of hypoxia/anoxia. Robust experimental data clearly showed that this ability relies on a number of evolutionary conserved strategies that, from genes to the whole organ, shape the cardiac response, allowing adaptation and organism survival also under conditions that may be severely detrimental for non-adaptable species. Special emphasis should be given to the recent acquisition of information regarding teleost fish genomes. Many gene products, among those annotated in the databanks and often present in duplicated forms, may represent novel physiological players and targets. In this respect, a thorough revision of the official annotations associated to the single sequences would be worth to favour the association of the proper gene products to the proper functions. As illustrated in the schematic overview we propose in Fig. 4, the nitrergic system is one of the most important players of the teleost cardiac response to low O_2. NO-dependent second messenger generation, as well as NO-mediated post-translational modifications such as S-nitrosylation and nitration, represent versatile signals that coordinate the activity of molecular mediators located in the biological domains of energy availability, gene expression, and cell homeostasis.

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CRediT authorship contribution statement

Sandra Imbrogno: Conceptualization, Writing – original draft. **Tiziano Verri:** Formal analysis, Visualization, Writing – original draft. **Mariacristina Filice:** Data curation, Visualization. **Amilcare Barca:** Data curation. **Roberta Schiavone:** Data curation. **Alfonsina Gattuso:** Writing – original draft. **Maria Carmela Cerra:** Supervision, Writing – review & editing.

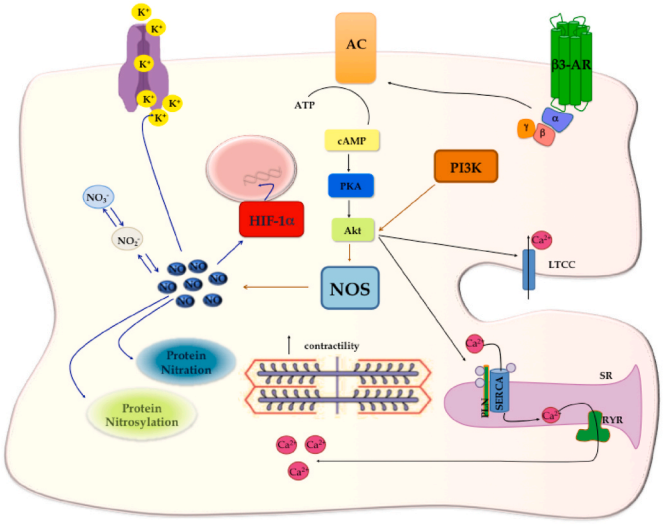

Fig. 4. Schematic overview of the NOS/NO-mediated intracellular pathways activated in fish cardiomyocyte under hypoxic stress. For details, see the text. AC, adenylate cyclase; AKT, serine/threonine kinase 1; β3-AR, beta 3 adrenoreceptor; cAMP, cyclic adenosine monophosphate; HIF-1α, hypoxia inducible factor-1α; LTCC, L-type calcium channel; NO, nitric oxide; NOS, nitric oxide synthase; NO^{2-}, nitrite; PI-3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PLN, phospholamban; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase pump; SR, sarcoplasmic reticulum.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphys.2022.03.006.

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