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## UNIVERSITÀ DEGLI STUDI DI TORINO

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# Anatomical features for the adequate choice of the experimental animal model in biomedicine: I. Fishes

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#### 14 ABSTRACT

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Fish constitute the oldest and most diverse class of vertebrates, and are widely used in 16 basic research because of a number of advantages (e.g., rapid development ex-utero, 17 large-scale genetic screening of human disease). They represent excellent experimental 18 models, to address studies on development, morphology, physiology and behaviour 19 function in other related species, and also informative analysis of the conservation and 20 diversity. Although less complex, fish share many anatomical and physiological features 21 with mammals, including humans, which make them an important complement to research 22 in mammalian models. 23

- In this review we describe and compare the most relevant anatomical features of the most used teleostean species in research, to be taken into consideration when selecting an animal model: zebrafish (*Danio rerio*), medaka (*Oryzias latypes*), the turquoise killifish (*Nothobranchius furzeri*), and goldfish (*Carassius auratus*).
- Zebrafish and medaka are the mainstream models for genetic manipulability and studies
   on developmental biology; the turquoise killifish is an excellent model for aging research;
   goldfish has been largely employed for neuroendocrine studies.
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#### 1 Introduction

Basic scientific research, defined as experimental investigative research to advance
knowledge without a specifically envisaged or immediately practical application, has been
put in contrast to applied research, which seeks specific solutions to targeted problems by
applying known fundamental results. This dichotomy should be overcome, since the two
types of research are inter-twined and interconnected, depending on each other. Both
basic and applied researches rely upon the use of animal models.

In basic research, mammalian models, such as rodents, have been pre-eminent in 8 modelling human physiological and disease processes, because of many anatomical, 9 physiological and genomic similarities, other than specific features (Lossi et al., 2015). 10 However, mammalian models could be disadvantageous in certain types of research. 11 Specifically, the large-scale genetic screening of human disease or developmental biology 12 could be hampered by murine long gestation period or by in utero gestation. The rapid 13 development ex-utero, allowing phenotypic analysis of embryogenesis and organogenesis 14 in vivo, the transparency of embryos and larvae, allowing the in vivo visualization of cell 15 biological events, the short lifespan made fish powerful and increasingly popular models in 16 17 basic research. Furthermore, Fish represent an unvaluable tool for comparative and evolutive approach and for studying various branches of biology, thanks to their 18 19 evolutionary position relative to those of other vertebrates, and to their high adaptive capacities. Fish constitute the oldest and most diverse class of vertebrates, comprising 20 21 around 48% of the known extant species in subphylum Vertebrata. Part of the wide variability they exhibit has been attributed to a whole genome duplication event (Sidow, 22 23 1996; Meyer and Schartl, 1999), which occurred at the base of the teleost radiation (Christoffels et al., 2004; Vandepoele et al., 2004). The duplication of a gene/genome led 24 25 to subsequent gene loss, to sub-functionalization or to neo-functionalization of the paralogs generated in the duplication event. After the initial genome duplication, the 26 genomes of different teleost lineages evolved independently. The independent evolution of 27 duplicated genes and the resulting sub-functionalization in fish can be useful for obtaining 28 results that are impossible to get for the corresponding (non-duplicated) homologue in 29 mammals (Furutani-Seiki and Wittbrodt, 2004). 30

Despite obvious differences, as fellow vertebrates, fish share many anatomical and physiological characteristics with mammals, including humans, which make them an important complement to mammalian models of disease. As a consequence, fish are used as experimental models to address function in other, often distantly related species, for

informative analysis of the conservation as well as diversity of processes that regulate
 development, morphology, physiology and behaviour (Lieschke and Currie, 2007).

In addition, scientists have been using Fish as substitutes of mammalian models in 3 biomedical research, also as consequence of the debate on the use of animal research 4 and the increasing awareness of the importance of humane use of vertebrates during 5 6 experimental procedures. Fish are excellent examples of application of the 3Rs principles (replacement, reduction and refinement), according to Russell and Burch (1959) in 7 research. Although fish display phenotypes resembling those of mammals (Santoriello and 8 Zon, 2012), they possess less complex anatomical and physiological features. 9 Furthermore, the great opportunity of conducting experiments on larval stages, when non-10 neuronal organs such as the heart are well developed, but the central nervous system 11 (CNS) remains relatively primitive, perfectly fits with the replacement concept. The 12 advantage of the size and transparency of fish larvae to perform similar procedures as 13 those performed in mammals represents a less invasive and more refined method. 14

This review discusses in detail the advantages of using fish as experimental model, 15 although fish diverged from humans more than 400-million years ago. Specifically, we will 16 describe commonalities to justify conducting research that is relevant to humans in these 17 animals, focusing on the main and relevant anatomical features of some of the most used 18 19 teleostean species in research, to be taken into consideration when selecting an animal model: zebrafish (Danio rerio), medaka (Oryzias latypes), the turquoise killifish 20 21 (Nothobranchius furzeri), and goldfish (Carassius auratus). Zebrafish and medaka represent the mainstream models for genetic manipulability (Schartl, 2014) and studies on 22 developmental biology; the turquoise killifish is an excellent model for aging (Cellerino et 23 al., 2015); goldfish has been largely employed mainly for neuroendocrine studies 24 25 (Popesku et al., 2008; Stacey et al., 2003). Turguoise killifish and medaka belong to sister groups, respectively Cyprinodontiformes and Beloniformes; zebrafish and goldfish, to the 26 order Cypriniformes. In this review we first give a short morphological description of each 27 species, and then we describe and compare the most relevant anatomical features of the 28 four species. 29

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#### 31 Fish species

Nothobranchius furzeri, also known as turquoise killifish, is a small freshwater fish, belonging to the order Cyprinodontiformes. It shows marked sexual dimorphism and dichromatism, and has a short maximum natural lifespan (<12 months) due to annual

desiccation of the pools in which they live. Notably, this short lifespan is also retained in 1 captivity, varying between 3 and 18 months (Cellerino et al., 2015). This peculiar 2 characteristic makes this killifish especially attractive for aging studies. The life cycle is 3 entirely adapted to the ephemeral and unpredictable conditions of their habitat. Fish hatch 4 when the pool is filled with water, grow rapidly and become sexually mature within few 5 6 weeks (Polačik et al., 2011). After reaching sexual maturity, they reproduce daily. When aged, at macroscopical level, animals display loss of color, mainly observed in males; loss 7 of body mass, which is reflected by a decrease in weight; curvature of the dorsal spine 8 (Genade et al., 2005) and deterioration of the fins, especially the caudal fin (Lucas-9 Sanchez et al., 2011). At microscopic level, progressive increases in granules of lipofuscin, 10 galactosidase and fluoro-Jade B in organs such as the liver, different areas of the brain 11 (Terzibasi et al., 2009), skin (Valenzano et al., 2006) and gills (Hsu and Chiu, 2009), 12 related to the cellular and tissue deterioration associated with aging. Furthermore, species 13 of genus Nothobranchius shows a high incidence of tumorigenesis, high frequency of the 14 onset of degenerative disorders in kidney and in liver (such as steatosis), in the heart 15 (fibrosis and aggregations of lymphocytes around myocardial fibres) and the gonads 16 17 (atrophy and fibrosis) (Lucas-Sanchez et al., 2014).

Oryzias latipes, also known as the medaka Japanese rice fish, is a small (2-4 cm long) 18 19 fish, one of the 27 species in the ricefish family Adrianichthyidae, in the order Beloniformes. It is closely related to other members of the superorder Acanthopterygii of 20 21 ray-finned fish such as pufferfish (tetraodon and fugu), stickleback and killifish, while it is separated from zebrafish by 150 million years of divergent evolution. Native to Southeast 22 Asia, the medaka lives in slow-moving streams, coastal tide pools and rice paddies. 23 24 Medaka are hardy fish that do well in a range of temperatures and water salinities; in the 25 wild they migrate between fresh and salt water areas, and are thus common along coastal 26 regions, in aquaria they prefer at least slightly brackish water.

Danio rerio, commonly known as zebrafish, is a small freshwater shoaling cyprinid fish, 27 originated from shallow ponds and standing water bodies, often connected to rice 28 cultivation in India. Zebrafish have fusiform, laterally compressed bodies that reach an 29 average length of 25 mm. They have centrally located eyes and thin elongate mandibles 30 with a protrusive lower jaw that causes the mouth to point upwards. Like other cyprinids, 31 zebrafish are stomachless and toothless. As a result, they rely on gill rakers to break up 32 food. Zebrafish have several defining features including an incomplete lateral line, two 33 pairs of barbels, and longitudinal stripes along the sides of their body. The degree of 34

sexual dimorphism in zebrafish is minimal, as males tend to have more yellow coloration
 and tend to have larger anal fins than females.

Carassius auratus, also known as goldfish, is a member of the freshwater family 3 4 Cyprinidae. There are several subspecies of *C. auratus*, all indigenous to Asia. The best known subspecies is *Carassius auratus auratus*, the common domesticated goldfish. The 5 6 common goldfish has two sets of paired fins - the pectoral fins and pelvic fins, and three single fins - the dorsal, caudal, and anal fin. They lack barbels on the upper jaw, and lack 7 scales on the head. Goldfish have exceptionally large eyes and acute senses of smell and 8 9 hearing. They have 27-31 scales along their lateral lines. Goldfish have (rather than true teeth) pharyngeal teeth in their throats, they use to crush food. 10

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#### 12 Genome

High-quality genome sequence and complete annotation of protein-coding genes of fish with identification of their human orthologues is essential to enhance the understanding of the detailed roles of specific genes in human diseases, both rare and common, and the use of fish as model in biomedical research.

- Data on all animal genome sizes are available on "Animal Genome Size Database" (http://www.genomesize.com). Although there are no significant different genome sizes among mammalian species, the situation changes considerably in fish species. For example, *Takifugu rubripes* (Aparicio et al., 2002) and *Tetraodon nigroviridis* (Jaillon et al., 2004) possess almost equal size of genome (400Mb), whereas medaka has 2-folds genome size and zebrafish 4-folds genome size (Imai et al., 2007).
- The genome of *N. furzeri* has been sequenced and assembled and will likely be released in the near future (Cellerino et al., 2015). The *N. furzeri* genome is 1.6–1.9 Gb in size and is characterized by a high repeat content (45%) (Reichwald et al., 2009). However, a comprehensive, annotated *N. furzeri* transcript catalogue and a first transcriptome-wide insight into *N. furzeri* aging, useful for functional studies of aging-related genes (Petzold et al., 2013), is now available.
- The medaka genome sequence project has been successfully completed (Kasahara et al. 2007) and the medaka draft genome is accessible through a number of genome browsers (<u>http://www.ensembl.org/Oryzias\_latipes/Info/Index</u>). The assembled genome of medaka includes 800 megabases. The small genome represents an advantage compare to zebrafish (Kasahara et al., 2007), for studies in developmental genetics, genomics and evolutionary biology.

Zebrafish genome has been sequenced (Howe et al., 2013). Detailed automatic and
manual annotation provides evidence of more than 26,000 protein-coding genes and,
compared to the human genome, shows that approximately 70% of human genes have at
least one obvious zebrafish orthologue (Howe et al., 2013).

The today goldfish is the artificial breed of the wild goldfish *Carassius auratus auratus* from 5 6 China. It shows diverse morphologies in body shape and coloration. Sofar, complete genome sequencing is not available, although numerous genes have been identified and 7 studied at expression levels (Unniappan et al., 2002). Molecular genetic markers, such as 8 random amplified polymorphic DNA (Suzuki et al., 2005); microsatellite DNA (Jorge et al., 9 2012); and partial sequences of mitochondrial DNA (Yamamoto et al., 2010; Cheng et al., 10 2012; Kalous et al., 2012) have been used for the identification of species, ploidy, clonal 11 lineages, and phylogenetic relationships. 12

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#### 14 Embryonic development

The advantage of using fish as model in vertebrate embryogenesis is also attributable to the external development of fish embryos from transparent eggs. The development is characterized by synchronous divisions, and absence of the G1 and G2 phases, thus cells proceed directly from the S to M phase (Graham & Morgna, 1966).

19 However, the developmental strategy of fish species is closely related with their life cycle. In annual fishes, such as *N. furzeri*, one-cell stage occurs at approximately 2 hours post 20 21 fertilization (hpf) (Hartmann and Englert, 2012). Cleavage lasts 75 min, and produces a typical teleost blastula (Iwamatsu, 2004) during the first day after fertilization. The gastrula 22 stage begins at day 2, and epiboly is completed at day 3. During epiboly, blastomeres 23 disperse and appear arranged in a striking pattern of near-uniform distribution. The 24 25 dispersion lasts for 5 days, and embryos might enter the first developmental arrest (diapauses I) (Hartmann and Englert, 2012). After re-aggregation, the neural keel forms, 26 and then somitogenesis and morphogenesis of the nervous system proceed until the 27 embryo enters diapause II. At this stage, the number of somites is fixed, the heart is 28 tubular and contractile, the major divisions of the encephalon are present and so are the 29 optic cups with the lens. The duration of diapause II is highly variable, from 2 days to up to 30 3 years, depending on the temperature (Valenzano et al., 2011). After diapause II, the 31 embryo proceeds to complete development. N. furzeri does not show diapause III under 32 standard laboratory incubation conditions. Hatching is a critical process for survival, also 33 because of a very hard chorion to digest (Cellerino et al., 2015). In laboratory conditions, 34

hatching can be induced by hypoxia (Levels et al., 1986). After hatching and under optimal
laboratory conditions, *N. furzeri* juveniles are immediately able to feed actively and show
rapid juvenile growth: the fastest maturation observed in a vertebrate with a typical
duration of 3–4 weeks, and one recorded case of 18 days from hatching to sexual maturity
(Blažek et al., 2013).

6 The embryonic development of medaka, under laboratory conditions, consists of 39 stages, with the latter corresponding to the hatching, and lasts about 9 days (Iwamatsu, 7 2004). The female medaka spawns between 20 and 40 eggs every day within a hour after 8 the onset of light. Fertilized eggs undergo cleavage, which takes approximately 30 min at 9 28.8 °C, gastrulation starts after 8.5 hpf and the neural axis is visible after 15 hpf. The 10 blastula period begins with a horizontal division in the central blastomeres, at 128 cell 11 stage the YSL begins to appear, at the end of this period the blastoderm has flattened 12 down capping the yolk sphere. The gastrula period begins at 20% of epiboly, rhythmic 13 contractions of the yolk occur and can be efficiently blocked by the addition of n-heptanol 14 to the medium without interfering with embryonic development (Rembold and Wittbrodt, 15 2004), thereby allowing extended observations and time-lapse video microscopy. This 16 17 period ends when epiboly is 90%, with appearance of brain rudiment, Kupffer's vesicle and optic vesicle. At stage of 2 somites, during segmentation, the epiboly is completed and 18 19 organogenesis occurs along the somitogenesis, lasting up to 140 hpf (Iwamatsu, 2004). Embryos hatch after 8-9 days at 28°C as fully developed juvenile fish, able to swimm and 20 21 feed.

The embryonic development of zebrafish begins about 40 minutes after fertilization. The 22 23 zygote period lasts up to 3-4 hpf, until the first cleavage occurs. After the first cleavage, blastomeres divide at about 15 minute intervals. During blastula period, lasting from about 24 25 2 to 5 hpf, cells continue to divide synchronously, the marginal cells collapse, and release 26 their cytoplasm and nuclei together into the immediately adjoining cytoplasm of the yolk cell. Thus the yolk syncytial layer (YSL) arises as prominent feature with Nomarski optics, 27 and important for staging. The YSL, an organ unique to teleosts, may be extraembryonic, 28 making no direct contribution to the body of the embryo. At first, the YSL has the shape of 29 a narrow ring around the blastodisc edge, but soon (within two division cycles) it spreads 30 underneath the blastodisc, forming a complete internal syncytium, that persists throughout 31 embryogenesis. In the late blastula, epiboly begins (Solnica-Krezel and Driever, 1994), as 32 the thinning and spreading of both the YSL and the blastodisc over the yolk cell. During 33 gastrula period, lasting about 5 to 10 hpf, epiboly continues, and in addition, the 34

morphogenetic cell movements of involution, convergence, and extension occur, 1 2 producing the primary germ layers and the embryonic axis. The segmentation period, from 10 to 24 hpf, is characterized by morphogenetic movements, and development of somites. 3 Furthermore, the rudiments of the primary organs become visible, the tail bud becomes 4 more prominent and the embryo elongates. Somites appear sequentially in the trunk and 5 6 tail, and provide the most useful staging index. Anterior somites develop first and posterior last. There are no transient somites in the zebrafish; the first somite forms the first 7 definitive myotome and so on. The pharyngula period (24-48 hpf), the time of development 8 when one can most readily compare the morphologies of embryos of diverse vertebrates, 9 corresponds to the second of the three days of embryonic development. The embryo 10 shows a well-developed notochord, and a newly completed set of somites that extend to 11 the end of a long post-anal tail. The nervous system is hollow and expanded anteriorly. 12 During the hatching period, from 48 to 72 hpf, depending also of the temperature, the 13 embryo continues to grow at about the same rate as earlier. Morphogenesis of many of the 14 organ rudiments is rather complete and slows down considerably, with some notable 15 exceptions including the gut and its associated organs. However, these endodermal 16 17 structures are difficult to visualize in the living embryo because of their deep positions. Much easier to see are the rapidly developing rudiments of the pectoral fins, the jaws, and 18 19 the gills. After 120 hpf (day 5), temperature dependent (Strähle et al., 2012), zebrafish become capable of swimming and feeding on external feed sources. 20

21 Finally, with regards to the embryonic development of the common goldfish, fertilized eggs are thick and not transparent, because of yolk texture, softer and larger compared to 22 23 zebrafish. At one-cell stage, perivitelline space appears and cytoplasm moves to animal pole to form the blastodisc (Tsai et al., 2013). The cleavage period starts 40 minutes 24 25 postfertilization and lasts up to about 3 hpf, forming 3 blastomeres layers. Blastula stages embryos are classified into high, oblong, sphere, and dome stages (Tsai et al., 2013). In 26 the high stage, blastodisc shows an elliptical shape. In the oblong stage, the border 27 between blastodisc and yolk is smooth and the blastomere shape remains elliptical. In the 28 sphere stage the shape becomes spherical or highly compressed pear-shape, and, finally, 29 in the dome stage yolk cell doming toward animal pole as epiboly begins (Tsai et al., 30 2013). The gastrula period last up to 12 hpf and is characterized by thickness of brain 31 rudiment, tail bud prominence. At this stage, epiboly is completed and embryos possess 32 one to five somites. The somitogenesis continues during the segmentation stage, which 33 completes at 22 hpf with 22 somites. The rate of somite appearance is approximately two 34

somites per hour in the goldfish. The pharingula period, lasting up to 44 hpf, is characterized by pigmentation in retina and skin, red blood cells on yolk, median fin fold with well extended actinotrichia, heart beat, pectoral fin bud appearance. Finally, in the hatching period, beginning 58 hpf and lasting up to 72 hpf, the pigmentation is completed, embryos display distinct yellow colored head and dorsal body. Moreover, at this stage there is a distinctive well-developed pre-cloacal median fin fold; pectoral fin is flattened and wide open mouth protruding anteriorly (Tsai et al., 2013).

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#### 9 Sex determination and differentiation

In fish, sex can be determined by mechanisms that are genetic, environmental, or a combination of both (Volff, 2005; Marshall Graves, 2008). Environmental factors that control sex determination in fish species include water temperature, density, and social interactions. Genetic control of sex determination is governed by the presence of sex chromosomes (visible sex chromosomes or heteromorphic chromosomes) that can be present either in males (XY) or in females (ZW).

- *N. furzeri* has a genetic sex-determination system, with males as the heterogametic sex,
   indicative of an XY/XX system (Valenzano et al., 2009).
- Medaka males and females differ by several secondary sex characters, some of which 18 19 such as the shape and size of the dorsal and anal fins - can be easily scored (Yamamoto, 1975). Additionally, strains with sex-linked pigmentation patterns have been 20 21 established and are available (Tomita et al., 1975; Handler et al., 1993). Morphological development of the gonads in all vertebrate groups appears to have been conserved 22 23 through evolution. Many genes that are important in gonadal sex differentiation in mammals, such as DMRT1, have been identified for the first time in medaka (Matsuda et 24 25 al., 2002; Nanda et al., 2002), and show gonad-specific expression during the period of sexual differentiation. The identified gene, mrt1bY, homologous of the mammalian, is the 26 male determining gene in the medakafish (Herpin and Schartl, 2011). Medaka has an XY-27 XX genetic sex determination system, with undifferentiated (homomorphic) sex 28 chromosomes (Matsuda et al., 2002; Nanda et al., 2002). It is the first vertebrate where 29 sex chromosomal inheritance and sex chromosomal crossovers have been described 30 (Aida, 1921). The first morphological sex difference of gonads appears in the number of 31 gonial-type germ cells one or two days before hatching (Iwamatsu, 2004). From this stage, 32 the activity of germ cell division in XX embryos becomes higher than that of XY embryos, 33 and then male germ cells arrest in mitosis (Kobayashi et al., 2004; Satoh and Egami, 34

1972). In males, somatic cells display an acinous structure, which is the precursor of the 1 2 testicular seminiferous tubules and can be distinguished at 10 days after hatching (Kanamori et al., 1985). In females, ovarian follicles are the first female-specific structure 3 4 and become evident around the diplotene oocytes about 20 days after hatching (Kanamori et al., 1985). After these structures have developed efferent ducts in the testes and 5 6 ovarian cavities in ovaries become apparent. Furthermore, unlike in higher vertebrates, full sex reversals can be obtained in medaka. Treatment with steroid sex hormones during the 7 larval period has generated YY males, XY females, XX males and even YY females 2. 8 Such experiments uncovered an important phenomenon: sex can be artificially reverted as 9 long as the gonad is morphologically indifferent (Wittbrodt et al., 2002). Finally, sex-10 specific pigmentation can be used to distinguish male from female embryos as early as 3 11 days postfertilization (dpf) (organogenesis stages) (Wada et al. 1998). 12

Zebrafish does not have a clear genetic basis of sex determination (von Hofsten andOlsson, 2005).

Goldfish possess an XX–XY sex determination system (Yamamoto and Kajishima, 1968). It is possible to control spawning, fertilization, and embryonic development by varying the water temperature and to manipulate both genetic and phenotypic sex by gynogenesis and temperature control (Yamaha et al., 1986, Yamaha et al., 1999, Goto-Kazeto et al., 2006).

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#### 20 Nervous system and sensory organs

Fish possess simple nervous systems compared to mammals, with far fewer neurons. However, they emerged as important model system for early patterning events and later events that build the three-dimensional structure of the brain. Genes that are important for fish brain patterning are usually conserved in mammals, and therefore relevant for understanding normal and abnormal human brain development and phisiology.

Fish brain is similar to other vertebrates, sharing many structural properties such as the 26 main organization (fore-, mid- and hind-brain, including diencephalon, telencephalon and 27 cerebellum), and the principal neurotransmitter systems. Despite smaller cerebral 28 hemispheres and the structure and function of the optic tectum compared to mammals, it 29 shows similarly defined areas such as the hypothalamus and olfactory bulb, encompassing 30 structures of the lateral pallium (located in the telencephalon), which appear to be 31 homologous to the mammalian hippocampus (Santana et al., 2012). However, it is also 32 well known that a great interspecific diversity exists in brain morphology in teleosts (Meek 33

and Nieuwhenuyis, 1998). This diversity offers large opportunity to correlate ecology with
 brains and sensory systems.

Turquoise killifish and medaka both belong to the superorder Acantopterygii, and their brain structures (D'Angelo, 2012; Ishikawa et al., 1999) are strikingly different in several important features from those of cyprinids, including zebrafish and goldfish (Wullimann et al., 1996; Peter and Gill, 1975).

The morphology of adult brain turquoise killifish has been described (D'Angelo, 2012), 7 showing a typical organization and subdivision of all teleosts central nervous system 8 (CNS). Particularly, it has been observed well developped visual system structures, e.g. 9 well prominent optic lobes, and glomerular nucleus. Also, glial cell population have been 10 identified (D'Angelo et al., 2012), with the preminent localization along the ventricles and in 11 the body of cerebellum. The brain is well suited for gene expression studies (D'Angelo et 12 al., 2014) and proteins (D'Angelo et al., 2014). In course of aging, typical cellular 13 phenotypes observed in the brain are: reduction of stem cells activity (Tozzini et al., 2012); 14 gliosis and neuronal degeneration (Valenzano et al., 2006). 15

- 16 The anatomy of medaka brain has been studied (Ishikawa et al., 1999), and the 17 morphogenesis has been studied based on a fate map and gene expression patterns 18 (Hirose et al., 2004; Kage et al., 2004; Ishikawa et al., 2008).
- 19 Zebrafish has proven to be an excellent model organism to study neurogenesis in the embryo (Schmidt et al., 2013; 2014). Studies have shown that the adult zebrafish brain 20 21 may also serve as a valuable model for the study of adult neurogenesis (Zupanc et al., 2005; Adolf et al., 2006). The brain of zebrafish is highly accessible during development, 22 because of the larval transparency. This allows in vivo neuronal network analysis. 23 24 Recently whole brain/single cell functional imaging techniques have been developed, 25 enabling monitoring of neuronal activity in hundreds of neurons at once (Leung et al., 2013). A detailed description of the brain of zebrafih during development is available on 26 27 http://www.zebrafishbrain.org/.

The description of CNS of goldfish represents one of the pioneering in a fish (Peter and Gill, 1975). The optic tectum is dome-shaped and continues to grow by the addition of sequential rings of new cells at the marginal portion called the peripheral growth zone (Raymond and Easter, 1983). Studies addressed to the localization of neurotransmitters and number of neuropeptides mainly related to neuroendocrine signaling have been carried out (Popesku et al., 2008) and represent a further useful tool for describing the morphology of brain structures and nuclei.

#### 1 Neuroendocrine system

The pituitary gland in fish secretes a number of hormones, which affect growth, osmoregulation, lipid metabolism and reproductive development and behavior, as well as controlling other endocrine glands (Bone et al., 1995). One major difference in the anatomy of the mammalian versus teleostean hypothalamo-pituitary axis is the median eminence, which connects the pituitary with the hypothalamus. In mammals is a stalk-like neurohemal structure, transporting neuropeptides, whereas in all teleosts this structure does not exist and the pituitary is positioned directly underneath the hypothalamus.

9 Any morphological descriptions are available on the pituitary gland of turquoise killifish.

10 The pituitary gland of medaka has accurately been described by Aoki and Umeura (1970). 11 It consists of three portions: the pars distalis, the pars intermedia and the 12 neurohypophysis. 8 different cytotypes have been characterized on the basis tinctorial and 13 histochemical properties. Transgenic models of medaka have been used to analyze the 14 multisynaptic neuronal circuitry regulating the pituitary functions (Karigo et al., 2014).

The pituitary gland of zebrafish consists of two different parts, which differ in 15 developmental origin and physiology. The neurohypophysis (posterior pituitary) derives 16 17 from a ventral extension of the developing hypothalamus and represents the neural compartment of the gland. It consists of axonal nerve endings from hypothalamic 18 19 magnocellular neurons, and pituicytes, which do not generate hormones but most likely have supportive and modulatory functions. Pituicytes can be readily identified by the 20 21 expression of specific marker genes such as fzd8b and and crap1b (Löhr and Hammerschmidt, 2011). The adenohypophysis (anterior pituitary) constitutes the 22 nonneural part and is embryologically derived from placodal ectoderm. It contains distinct 23 endocrine cell lineages, which are characterized by the type of hormone they secrete. Nine 24 25 different cell types can be distinguished on the basis of anatomical position and hormone expression profile: lactotropes, two distinct groups of corticotropes, thyrotropes, 26 somatotropes, two groups of somatolactotropes, melanotropes, and gonadotropes (Löhr 27 and Hammerschmidt, 2011). Zebrafish represents a powerful approach to elucidate 28 developmental and physiological mecha- nisms of the endocrine system. Classical gain-of-29 function approaches like intraperitoneal and intracerebroventricular hormone injections, 30 although possible, are technically much more challenging. For this purpose goldfish is 31 32 more suitable.

Goldfish, indeed, has been using as an excellent model for neuroendocrine regulation of energy balance and reproduction (Bernier and Peter, 2001a; Popesku et al., 2008). The

pituitary gland consists of three lobes: rostral and proximal pars distalis, and the neuro-1 2 intermediate lobe. The rostral pars distalis is the smallest of the three lobes, occupies the dorsal area of the posterior part of the gland and is intimately related to the posterior 3 aspect of the short and delicate pituitary stalk. It contains acidophilic, basophilic and 4 chromophobic cells. The proximal pars distalis, the second pituitary lobe, was found 5 6 beneath both the rostral pars distalis and the pituitary stalk. It extended to a varying degree into the anterior part of the gland and contained two types of basophilic and one 7 type of acidophilic cell. In the largest pituitary lobe, the intrinsic cells did not exhibit 8 conspicuous staining properties (Kaul and Vollrath, 1974a,b). Gonadotroph cells are 9 clustered in the proximal pars distalis in association with somatotrophs (Ball, 1981), which 10 allows for the precise determination of the preoptic telencephalic and hypothalamic origins 11 of hypophysiotropic inputs to he pituitary using tract-tracing methods (Anglade et al., 12 1993). The anterior pituitary gland is innervated by numerous neuronal cell types, and thus 13 pituitary hormone release is directly regulated. Thanks to regionalized distribution of cells 14 in the goldfish pituitary, it has been demonstrated a unique reciprocal paracrine 15 relationship between gonadotrophs and somatotrophs, mediated by luteinizing hormone 16 and growth hormone (Wong et al., 2007). 17

18 Sensory organs

19 The retina of teleosts consists of three nuclear layers and two plexiform layers. The outer nuclear layer contains the cell bodies of the photoreceptors (rods and cones). The inner 20 21 nuclear layer contains the cell bodies of the horizontal, bipolar and amacrine cells, and the ganglion cell layer contains the ganglion cell bodies. The plexiform layers are found 22 23 between the nuclear layers, where the synaptic connections between the retinal neurons take place. The outer plexiform layer (OPL) consists of the connections among 24 25 photoreceptors, bipolar and horizontal cells, and the inner plexiform layer (IPL) consists of the connections among bipolar, amacrine and ganglion cells (Bilotta and Saszik, 2001). 26 Few differences, ascribable to organization of cones and rods, have been described 27 between medaka and zebrafish (Tohya et al., 2003). Since retina is a well anatomical 28 conserved structure in Teleosts, zebrafish (Hoon et al., 2014), medaka (Conte et al., 29 2010), the turquoise killifish (Gatta et al., 2014) and goldfish (Braisted et al., 1994) have 30 been using as models for addressing studies on development, physiology of visual system 31 and perception (Rosa Salva et al., 2014), regeneration (Goldman, 2014), and diseases 32 33 (Hoon et al., 2014).

34 Olphactory system

Fish olfactory system comprises only one main pathway, originating in the nasal cavity and giving rise to what is commonly referred to as the main olfactory system in mammals. Fish lack the other pathway, originating in the vomeronasal organ and giving rise to the accessory olfactory system (Dulka et al., 1993).

The nasal cavity of zebrafish is displaced by the nasal pit in zebrafish, a tubular structure. 5 6 that opens to the exterior via anterior and posterior pores and has no communication with the oral cavity (Taniguchi and Taniguchi, 2014). In the nasal cavity, there are bilaterally, 7 symmetric olfactory epithelia (OE) that are folded into rosette-shaped sensory organs. 8 Each OE is connected via a short olfactory nerve to the olfactory bulbs. Three types of 9 olfactory receptor neurons have been identified in the OE: ciliated, with round somata 10 located deep in the epithelium and extended long, ciliated dendrites to the epithelial 11 surface (Castro et al., 2006; Gayoso et al., 2011); microvillous, with various morphologies 12 and differences in antibody labeling at intermediate depths in the OE; and cryptic, with 13 ovoid shape, rounded apical pole, eccentric basal nucleus, and located near the surface of 14 the sensory epithelium, labeled, among others, by S100 (Braubach et al., 2012; Parisi et 15 al., 2014). Axons of olphactory neurons target the glomeruli in the olphactory bulbs. 16 17 Glomeruli are organized in nine distinct regions reproducibly located on dorsal, ventral, lateral, and medial surfaces of the olfactory bulbs (Braubach et al., 2012). 18

19 The olfactory system of goldfish includes anatomical and functional subdivisions, that 20 resemble those associated with the main and accessory olfactory systems in tetrapods, 21 being particularly well suited for comparisons. The main reasons are:

the olfactory pathways that regulate responses to sex pheromones in goldfish are
 different from those that serve a more general olfactory function;

the functional differences seem to be subserved by separate and anatomically distinct
 olfactory tract projections to the brain;

the lateral olfactory tracts and their central projections in goldfish appear to serve a
function analogous to that of the main olfactory system, while the medial olfactory tracts
and their central projections comprise a pathway similar to the vomeronasal-accessory
olfactory system (Dulka, 1993).

30 Lateral line system

In fishes and amphibians, the lateral line is a superficial mechanosensory system, combining some structural and physiological characteristics of the mammalian vestibuloauditory and somatosensory systems. Lateral line may be complete, running from the head to the tail, or incomplete, starting at the head and ending before the tail.

In zebrafish, lateral line is a model for studying the coordination of cell migration and 1 2 morphogenesis, in addition to its use for studying hair cell biology relating to human hearing and balance disorders (Whitfield, 2002; Nicolson, 2005). Lateral line system is 3 made of peripheral receptors, mechanoreceptive neuromasts containing mechanosensory 4 hair cells innervated by afferent and efferent neurons and surrounded by nonsensory 5 6 support cells (Ghysen and Dambly-Chaudière, 2007; Bleckmann and Zelick, 2009). Hair cells locally acquire mechanical signals and transform it into chemical signals that are 7 further converted into electrical impulses, transported to the brain by afferent neurons. Hair 8 cells possess a mechano sensing organelle protruding from the cell's apical surface. In the 9 neuromast, hair cells are contained within a gelatinous cupula that projects into the 10 surrounding water, and are formed by an array of stereocilia arranged in rows of 11 increasing length, and a kinocilium eccentrically located adjacent to the tallest stereocilia. 12

13

#### 14 Cardiovascular system

- In fishes a single heart circuit causes the blood to be directly routed through the entire
   organ in a posterior to anterior direction. In order, the primitive heart chambers are:
- 17 1) sinus venosus, a thin-walled distensible sac into which the venous blood is returned;
- 18 2) atrium, also thin-walled;
- 19 3) ventricle, the thick-walled major contractile portion of the heart;

4) conus arteriosus, a thick, but narrow tubular portion of the heart that is continuous with 20 the ventral aorta. Hearts of fish, from the perspective of myocardial oxygen supply, have 21 four main arrangements. The type I has entirely spongy myocardium and a cardiac 22 23 circulation. The type II heart has an outer compact myocardium separated from the spongy myocardium by a layer of connective tissue. The coronary vessels in the compact 24 25 myocardium do not penetrate the spongy myocardium. The type III and IV differ from the type II hearts because the connective tissue lacks and coronary vessels penetrate the 26 spongy myocardium. 27

Any morphological data are available on the heart of turquoise killifish.

The heart morphology of medaka was studied by light and electron microscopy (Lemanski et al., 1975). The epicardial layer forms an outer covering over the organ and is composed of simple squamous epithelial cells. The ventricle is trabeculated, showing a "spongy" appearance; the atrium is less extensively trabeculated. The myocardial cells of the trabeculae have small diameters but extend for considerable distances. The myofibrils usually are located peripherally, while the nucleus, mitochondria, and other cellular organelles are located centrally. The endocardium is composed of a continuous layer of
 cells that appear to be metabolically very active (Lemanski et al., 1975).

The cardiac morphology of developing (Hu et al., 2000) and adult (Hu et al., 2001) 3 4 zebrafish has been described. Furthermore, several studies have been conducted to validate zebrafish as model for cardiovascular disease and vasculogenesis (Asnani and 5 6 Peterson, 2014). The use of fluorescent reporters has been essential to identify two discrete phases of cardiomyocyte differentiation necessary for normal cardiac 7 development in the zebrafish. These phases are analogous to the differentiation of 8 cardiomiocytes in mammals, thus heart embryogenesis is conserved between zebrafish 9 and mammals (Asnani and Peterson, 2014). Zebrafish has been established as model for 10 discovering molecular mechanisms of human cardiovascular diseases, which includes 11 prevalent forms of cardiomyopathy: dilated cardiomyopathy and hypertrophic 12 cardiomyopathy. Many of the implicated genes in human cardiomyopathy such as titin (ttn) 13 (Xu et al., 2002), tropomyosin (tpm4) (Zhao et al., 2008), troponin 2 (tnnt2) (Sehnert et al., 14 2002), myosin light chain (cmlc1, myl7) (Rottbauer et al., 2006) and myosin heavy chain 15 (myh6) (Berdougo et al., 2003) have been mutated also in zebrafish, and causing 16 cardiomyopathy, revealed by ultrastructural examination (Poon and Brand, 2013). 17

Goldfish possesses a type II heart that consists of a relatively thin vascularized compact
 heart and an extensive avascular spongy heart. In addition, goldfish displays a more
 saccular shaped heart, perhaps reflecting their specific ecological physiology (Grivas et al.,
 2014).

22

#### 23 Urinary apparatus

The kidney in fish is located retroperitoneal, exterior to the dorsal wall of the body cavity. 24 25 The kidney is a paired organ that has been described as having various anatomical and functional compartments (Morovvati et al., 2012). The kidney of fish receives majority of 26 postbranchial blood and renal lesions may be expected to be good indicators of 27 environmental stress. The head of kidney contains endocrine elements, the chromaffin 28 cells and interregnal tissue, which are located around the blood vessels. The posterior 29 kidney contains the nephrons with variable quantities of hemopoietic and lymphoid tissue 30 in the interstitium. 31

In the kidney of medaka, the glomeruli are frequently found beneath the renal capsule, which consisted of fine connective tissue. Like mammals, each medaka glomerulus

exhibited a well developped glomerular capillary and an arborized mesangium in medaka
 adult (Ichimura et al., 2013).

There are several advantages in studying glomerular development in the medaka 3 4 pronephric glomerulus compared to zebrafish and other teleosts (Ichimura et al., 2012). The glomerular primordium of the medaka pronephros exhibits a C-shaped epithelial layer. 5 6 The C-shaped primordium contains a characteristic balloon-like capillary, which later 7 divides into several smaller capillaries. A pair of pronephric glomeruli remains independent of each other due to the interposition of the mass of interglomerular mesangium (IGM) 8 between them. The IGMCs possesses numerous cytoplasmic granules throughout 9 pronephric development. The morphological process of podocyte differentiation in medaka 10 is more similar to mammals (Ichimura et al., 2012). In particular, the glomerular 11 primordium of the medaka pronephros exhibits a C-shaped epithelial layer of primitive 12 13 podocytes, which is similar to that of mammalian S-shaped body.

The adult zebrafish kidney, or mesonephros, is a single, relatively flat organ attached to the dorsal body wall that consists of characteristic bilaterally symmetric regions referred to as the head (or anterior), trunk (or medial), and tail (or posterior) (Gerlach et al., 2011).

17

#### 1 **Table 1.**

Main field of use Anatomy and physiology Applied pharmacology		Turquoise killifish Basic research (age research)	Medaka Basic research (developmenta l biology)	Zebrafish Basic research (developmental biology)	Goldfish Basic research (neuro- endocrine studies)
		Pathology	Cancer and cancerogenesis	Spontaneous tumors in brain, liver and genital apparatus (Di Cicco et al., 2011)	Spontaneous lymphoma, ovarian tumour; induced melanoma, liver tumors, and xenograft (Hasegawa et al., 2009)
	Toxicology		Ecotoxicity/toxi city tests (Padilla et al., 2009)	Ecotoxicity/ drug development (Gaytán and Vulpe, 2014)	Ecotoxicity (Velma and Tchounwou, 2011)
	Neuro- degeneration and neuropathology	Neuro- degeneration (Valenzano et al., 2006)	Retinite pigmentosa (Conte et al., 2015)	Genetic-based pathologies (Newman et al., 2014)	
	Endocrinology and endocrine pathologies			Diabetes. Food intake regulation (Matsuda et al., 2012)	Hypothalamic- pituitary axis. Food intake regulation (Popesku et al., 2008).
	Genetic pathologies		General use (Schartl, 2014)	General use (Schartl, 2014)	
	Others			Cardiovascular diseases (Asnani and Peterson, 2014).	

\* melanoma, pancreatic tumors, T cell lymphoma or leukaemia, B cell leukaemia, rhabdomyosarcoma, neuroblastoma, lipoma, Ewing's sarcoma, myeloproliferative neoplasms, corticotroph adenoma and neoplasm, testicular germ cell tumour.

2 A summary of the utilization of different fish species in current biomedical research.

**Table 2.** 

	Turquoise	Medaka	Zebrafish	Goldfish
	killifish			
	Hatching 12 days	Hatching9 days	Hatching 3 days	Hatching 3 days
a	after fertilization, as	after fertilization,	after fertilization,	after fertilization,
Embryonic ii	ndipendent feeding	as indipendent	indipendent	indipendent
development	larva	feeding larva	feeding after 5	feeding after 5
			days post	days post
			fertilization	fertilization
Digestive	Oral and	Oral and	Teeth attached to	Pharyngeal teeth.
apparatus	pharyngeal teeth.	pharyngeal	the fifth branchial	
		teeth.	arch.	Stomachless
		Stomachless	Stomachless	
	Stomach			
Reproductive S	exual dimorphism	Sexual	Sexual	Sexual
system		dimorphism	dimorphism	dimorphism
Skin	Highly colourful	Pigmented skin.	Five uniformly,	Head without
	skin in male.	4 types of	pigmented,	scales
		cromatophores	horizontal stripes	
			on the side of the	
			body	
Lateral line C	complete	Subdivided in	Subdivided in	Complete
		anterior and	anterior and	
			n a a ta ri a r la ta ral	
		posterior lateral	posterior lateral	
		line, according to	line, according to	
		line, according to the neuromasts	line, according to the neuromasts	

General anatomical features of the most used fish in biomedical research.

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