SCUOLA NORMALE SUPERIORE

PISA

CLASSE DI SCIENZE MATEMATICHE, FISICHE E NATURALI

Corso di perfezionamento in Neurobiologia

Triennio 2003-2005

Aging in *Nothobranchius furzeri*, a new Vertebrate Model of extremely Short Lifespan.

Marzo 2006

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Chapter 1

Introduction

1.1 The Biological Concept of Senescence

Biological senescence (or aging) is a general biological process which consists in the age-dependent global decline in life variables associated with individual fitness, such as the ability to reproduce, to respond to stress and to maintain homeostasis. Senescence begins after development and occurs in almost all eukaryotes, in fact only a negligible number of species are reported not to senesce at all (Finch, 1990). Senescence is associated with an age-dependent progressive increase in the risk of disease as well as with an exponential increase in population death rate.

The mechanisms responsible for aging are not completely understood but there is large agreement about the role of Reactive Oxygen Species (ROS) as principal actors during the aging processes (Tuma, 2001). Reactive Oxygen Species are mostly iproduced during mitochondrial oxidative phosphorilation and cause damages in nucleic acids (especially mitochondrial DNA), proteins and lipids. Respiration (oxygen consumption) and

ATP synthesis are tightly linked to the production of the damaging ROS, i.e metabolic energy conversion generates by default potentially damaging factors. The capacity to prevent or repair the damages produced by ROS is usually highly efficient in young organisms but starts to decline after development and sexual maturation, causing accumulation of damage at cell and tissue level. Interestingly, studies on cell cultures from different mammal species have shown that long-lived species are better in repairing tissue damages induced by multiple stressors compared to short-lived ones (Kapahi et al., 1999), suggesting a possible role of repairing mechanisms as checkpoints for the initiation and progression of senescence and determination of longevity.

Another mechanism generally associated to aging is telomere shortening, a process occurring in replicating somatic cells which consists in the progressive erosion of chromosome's end regions due to mitotic non-homologous recombination of highly repetitive sequences which eventually leads to chromosome instability and can induce cell cycle arrest, apopotosis and neoplastic transformations. Telomere shortening can be rescued by the ribonucleoprotein complex *telomerase*, which is normally inactive in somatic cells and exerts its action in germline cells, stem cells and cancerous cells.

Generally two classes of senescence are recognized: *Cellular Senescence* and *Organism Senesence*. *Cellular Senescence* consists in the irreversible withdrawal of the cell from the cell cycle, i.e. the cell stops undergoing mitosis without necessarily undergoing apoptosis but losing the capacity of regenerating and self-renewing. More globally, *Organism Senescence* consists in a generalized loss of functional efficiency (physiological decline) of all organism's biological functions which eventually causes death. It is debated whether *Organism Senescence* is the outcome of synchronous senes-

cence of different tissues, the cumulative (cascade) effect of failures occurring asynchronously in several tissues independently, or whether it is centrally controlled by one or more diffusible factors, a central hormonal *aging clock* which gives the pace to the organism senescence. This is the case in some plants, where specific organ senescence (possibly due to apoptosis) is genetically programmed (Hadfield et al., 2000), or sudden death is achieved after flowering, like in bamboo (Keeley and Bond, 1999). In an analogous fashion, pacific salmom, after reproduction, undergoes sudden death (Dickhoff, 1989). Metazoa, and in particular vertebrates, have –at the same time– tissues with different replicative states, e.g. fibroblasts, blood cells, muscle fibers, hepatocytes, etc. are replicative active throughout adulthood, whereas most neural cells,[1] after neural system development, are mainly post-mitotic cells with low (if any) regenerative potential, although still able to undergo plastic modifications. This view of a post-mitotic brain, has been recently challenged by the evidences of the presence of different sources of adult neural stem cells (Meletis et al., 2006). These differences in regenerative potential among tissues (*in vivo* studies) suggest diverse classes of failure dynamics: replicating tissues would accumulate nuclear-DNA and mitochondrial-DNA mutations due to errors in DNA replication, high chance of neoplastic transformations following telomere erosion and consequent cell cycle instability. This can explain the mechanism of cellular senescence as a defense against cancer progression, which has recently been demonstrated *in vivo* (Collado et al., 2005). Post mitotic cells, like neural cells, which have high metabolic activity, would accumulate ROS-dependent damages, like protein misfolding, lipid peroxydation and mtDNA mutations.

For virtually all tested model organisms, manipulations of nutritional

[1] expecially central nervous system neurons

state indicators, either through a *Dietary Restriction* protocol or negatively interfering with the intra-cellular signalling in the Insulin/IGF-1 (Insulin like growth factor 1) pathway, are sufficient to induce a significant increase in lifespan and to retard the onset of several aging markers (Tatar et al., 2003). This means that the relationship between food intake (usually captive models are fed *ad libitum*) and aging rate, does not necessarily depend on the amount of food assumed, but on the food-dependent activation of the Insulin/IGF-1 pathway. Since aging rate and life expectancy are natural traits dramatically important for organisms' fitness and for population success in given ecological conditions, it is likely that the Insulin/IGF-1 signaling is subjected to intense selection.

1.2 Longevity

Longevity, often measured as "maximum life expectancy", is a natural trait which shows high variability in natural populations: from 10-15 days (few tens of cell divisions) in yeasts and protozoans to over 5000 years in Bristlecone pines. Longevity in natural populations is generally reported in association with a wide set of variables, like body mass, metabolic rate, energy metabolism, duration of development and sexual maturation, encephalization index, number of offspring produced lifelong, efficiency in cell damage repair.

Larger species usually live longer than smaller species[2] and have lower metabolic rates than shorter living ones, but this latter correlation has been proven to be strongly biased by the correlation between body mass and measures of metabolic rate, i.e. the residuals of the correlation of metabolic rate and maximum lifespan to body mass are in fact not correlated with each other (Speakman, 2005b).

Energy metabolism is importantly correlated to lifespan within a clade (i.e. considering separately mammals and birds) and also within a species, in fact, in species with strong coupling of oxygen reduction and ATP production, with consequent formation of damaging Reactive Oxygen Species, maximum life expectancy is low. Conversely, in species with strong uncoupling of oxygen consumption and ATP production, even with high ATP production, maximum life expectancy is higher (fewer Reactive Oxygen Species are produced) (Speakman, 2005a). Interestingly, mice with higher metabolic rates and higer mitochondrial uncoupling live longer than those, in the same

[2] an opposite trend is observed within a species, i.e. larger dogs, mice, flies (depending on temperature) and people, live less than smaller ones (Speakman et al., 2003; Miller et al., 1999; Norry and Loeschcke, 2002; Samaras and Elrick, 2002).

cohort, with lower metabolism and uncoupling (Speakman et al., 2004). In average, birds, compared to mammals, combine longer lives with higher metabolism (Speakman, 2005a).

The time required to complete development and reach sexual maturity, often measured as "reproductive age", has been shown to be correlated to lifespan in several taxa (Martinez, 1998; Charnov, 1993); moreover, flies selected for late reproduction also lived longer than flies selected for early reproduction (Sgró et al., 2000).

In haplorhyne primates[3] there is a positive correlation between brain mass and lifepan even when the effect of body mass is removed (Allman et al., 1993).

There is an inverse correlation between the log of maximum number of offspring produced under ideal conditions and maximum lifespan, with highly productive species living less than species with low offspring production (Holliday, 1997). An exception in this trend is the temperate bear (*Ursus*), which has more offspring than what is expected by its maximum lifespan. Temperate bears undergo hibernation, a physiological adaptation often related to slowed aging in many animal models (e.g. hamsters and squirrels), which is correlated to longer lifespan than what is expected from the body mass. Interestingly, hibernation has been suggested to share cellular mechanisms with calory restriction (Walford and Spindler, 1997).

As mentioned above, long-lived organisms have been shown to have better mechanisms of cell damage repair compared to short-lived ones. Holliday (Holliday, 1997) reports studies in which aging-related biomarkers (e.g. longevity of fibroblasts and erythrocytes *in vitro*, cross-linking of collagen, auto-oxidation of tissues, etc.) where quantified in "long-lived"(humans)

[3]tarsiers, monkeys, apes and humans.

and "short-lived" (mice and rats) mammals, the former having higher efficiency in cell maintenance functions than the latter. This same relationship holds when the number of species is increased, as shown by Kirkwood (Kapahi et al., 1999), who showed that longer living species share higher in vitro cell survival than shorter living ones.

1.3 Theories of Aging

Aging and longevity are natural traits strictly related to survival and therefore importantly linked to natural selection. In the XIX century, Alfred Russel Wallace, the co-discoverer of "Natural Selection" as the principal mechanism by which life forms evolve, first noted the important link between lifespan and death by one side and natural selection by the other:

> ... when one or more individuals have provided a sufficient number of successors they themselves, as consumers of nourishment in a constantly increasing degree, are an injury to those successors. Natural selection therefore weeds them out, and in many cases favours such races as die almost immediately after they have left successors.

but it was later August Weismann who highlighted on the importance of evolution for the understanding of aging (see later in this section).

Why do (almost) all organisms age? Does natural selection selects for death programs? In other words is longevity programmed? Is aging independent from development? The core of the aging-problem stems in the observation that the failures seen in aging occur after maturation. As observed by George Williams (Williams, 1957)

> ...it is astounding that a complex animal, after completing the almost miraculous feat of embryogenesis, fails at the seemingly much easier task of simply preserving what was already created.

This is indeed even more striking when one thinks that *there is no physical reason why living organisms cannot sustain themselves at full, youthful*

vigor for an indefinite period of time,[4] in fact the second law of thermodynamics[5] applies to closed systems, whereas organisms get free energy from the sun and/or the food and release entropy into the environment. In few words, organims exchange energy with the environment and could possibly be able to maintain the soma, despite the accumulation of errors which inevitably occur during life processes like DNA replications, natural exposition to stressors and mutagenic agents. The failure of repairing systems is responsible for the "wear and tear", i.e. structural damage due to stochastic failure accumulation, which reduces system's redundancy and is the principal cause of global failure in organisms and technical devices (computers, vehicles, etc.), which have also been shown to undergo "aging" but in a different fashion from living organisms (Gavrilov and Gavrilova, 2003).[6]

In general, living organisms, unlike technical devices, are self organizing systems,[7] able to build and maintain themselves, to self repair and regenerate tissues and, in some cases, organs (at least for a given period of their life) until at some point of their life such maintenance mechanisms fail and organisms start to loose vigor, senesce and eventually die. The way in which organisms undergo senescence varies greatly among species, in fact, considering senescence by age dependent death rate increase, we observe species with death kinetics ranging form almost "no increase" like hydra and some turtle species (Martinez, 1998; Miller, 2001), to those with "gradual rates", like humans. Other species, like the pacific salmon, show very rapid, almost "discontinuous increase" in death rate (Dickhoff, 1989).

[4] J. Mitteldorf, personal communication.

[5] in a closed system entropy (a measure of disorder) will increase.

[6] technical devices follow Weibull (power) law in failure dynamics, whereas organisms follow Gompertz-Makeham exponential law.

[7] Autopoiesis is necessary and sufficient to characterize a living system (Cohen and Wartofsky., 1980).

From the above analysis comes out that the research on aging requires a theoretical framework for answering to the following important questions

- Why living organisms lose the capacity to self maintain themselves?

- What determines the onset of aging?

- Which mechanisms are responsible for the different observed aging rates?

which are the core of modern gerontology and which can provide us a tool for interfering with the aging process and for understanding how to deal with age-associated diseases.

1.3.1 Mutation Accumulation Theory

In the middle of the XXth century Sir Peter Medawar formulated the *Mutation Accumulation Theory of Aging*, which considers aging as a non-adaptive byproduct of natural selection (Medawar, 1952). In this view, selection cannot counteract the effects of deleterious alleles expressed in the post-reproductive life since such effects are not negative for fitness. These "late expressed bad alleles" therefore would accumulate throughout generations and globally cause the ensemble of aspects which we collectively call aging. In line with this hypothesis, it makes sense that gene variants responsible for progerias (pathological premature aging) are wiped out by selection and therefore are very rare in population, whereas those which contribute to

Hungtington disease, whose symptoms raise in elders, are much more frequent because they are not eliminated by selection. This is indeed what is observed (Le Bourg, 2001). Although some experimental evidence suggest that *Mutation Accumulation Theory of Aging* works in *Drosophila* (Hughes et al., 2002), it is not clear yet how the exponential increase in death rate induced by alleles with late-life detrimental effects would decrease at very old ages, in the so-called late-life mortality deceleration observed in many species (Gavrilov and Gavrilova, 2003). According to the *Mutation Accumulation Theory of Aging*, organisms in post-reproductive ages are selectively neutral, since they have already transmitted their genes to the offspring. From a population genetic perspective this is not necessarily correct, in fact high population growth rate and low extrinsic mortality induce overcrowding and high competition for food resources in isolated populations with a limited amount of food resources. In these special cases, alleles with expression in late life can be selectively important (see later subsection "Adaptive Programmed Death").

The expectation that random mutations would accumulate in genes with late life phenotypes is in contradiction with the recent observations of the mechanisms of aging across taxa is highly conserved (Guarente and Kenyon, 2000) and casts serious doubts on the validity of the *Mutation Accumulation Theory of Aging*.

1.3.2 Antagonistic Pleiotropy Theory

George Williams, known for being among the fathers of the gene-centric view of evolution, postulated in 1957 that genes with a late detrimental effect contributing to aging coud be positively selected if they have early life

positive effects: (Williams, 1957)

> Senescence might be regarded as a group of adptively unfavorable morphogenetic changes that were brought in as side effects of otherwise favorable genes, and which have only been partly expurgated by further selection. There are therefore, two opposing selective forces with respect to the evolution of senescence. One is an indirect selective force that acts to increase the rate of senescence by favoring vigor in youth at the price of vigor later on. The other is the direct selection that acts to reduce or postpone the "price" and thereby decrease the rate of senescence. The rate of senescence shown by any species would depend on the balance between these opposing forces.

Following such view, there could be a positive selection for senescence when the effect on fitness of some allele is *antagonistic*. In the previous "Mutation Accumulation Theory", instead, late acting alleles with a negative weight on fitness, accumulate without the action of selection. From this "Antagonistic Pleiotropic Theory" Williams produced nine testable deductions, among which two, in particular, were largely cited and tested and inspired a remarkable amount of scientific work worldwide:

- *Rapid individual development should be correlated with rapid senescence*

- *Successful selection for increased longevity should result in decreased vigor in youth*

The first of the two quoted deductions highlight the correlation between duration of development and senescence rate. This correlation was tested in populations of *Drosophila* and no evidence was found supporting any relationship between these two life-history traits (Economos and Lints, 1985).

The second expectation gave rise to several tests of artificial selection for long life, of which the most complete was conducted by Michael Rose who, for more than 12 years since 1980, was selecting *Drosophila* populations for longevity (Leroi et al., 1994). He found, in contradiction with Williams' theory, that fertility was not reduced in long-lived selected lines which, unexpectedly, were more productive[8] than the original non-selected strain. Although it was elegantly shown that genes involved in aging have wide cytological connectivity, i.e. are significantly above the chance level of cell metabolic network connectivity (pleiotropy) (Promislow, 2004), it is also true that Williams prediction that senescence is

> ... never due largely to changes in a single system

is against more recent findings that single gene mutations (e.g. in the Insulin/IGF-1 like pathway) are able to extend lifespan in several model systems (see later).

"Antagonistic Pleiotropy Theory" has recently been used as a theoretical framework for explaining the effects of tumor suppressive mechanisms evolved to prevent the development of cancer in young organisms (Campisi, 2005a; Campisi, 2005b). In fact, organisms with renewable tissues evolved mecanisms of tumor suppression which are beneficial early in life, reducing the risk of tumor onset, but are deleterious late in life, contributing to the arrest of cellular proliferation and eventually affecting tissue renewal, repair

[8] per day egg deposition.

and regeneration (Campisi, 2005b). This view requires that the activity of tumor suppressor genes acting during development would be correlated to that acting during adulthood and elderly, in order to achieve, with the same tumor suppressor system, different (antagonistic) effects at different ages. To my knowledge there is no evidence supporting this prediction and it would be interesting to investigate in such direction, in order to validate the antagonistic pleiotropic action of tumor suppressor genes.

1.3.3 Disposable Soma Theory

A more recent variant of "Antagonistic Pleiotropy Theory" underlines the importance of the energetic trade-off between soma-maintenance and reproduction for the onset of a complex aging phenotype. Thomas Kirkwood (Kirkwood, 1977) postulated that metabolic resources allocation between soma-maintenance and reproduction is optimized in order to maximize individual fitness. According to this theory, aging starts just after sexual maturity, when energy requirements for reproduction induce a depletion in available energy for the maintenance of the soma and, consequently, a progressive physiological failure

> ... senescence ... as a change in state that depends on the resources allocated to maintenance (Shanley and Kirkwood, 2000).

Pleiotropic effect is achieved when genes selected for fast growth and high-rate reproduction are the same to have a late-life negative phenotype (e.g. hyperproliferation favoring cancer onset). This can be generally disadvantaging, but, if the chances to survive are low, it pays more (in terms of fitness) to have fast reproduction, than to keep a functional soma at the

expenses of reproduction, i.e. with high extrinsic mortality it would be therefore useless to invest too much in the soma maintenance.

Disposable Soma Theory expectations about a clear trade-off between soma and reproduction have not been yet confirmed. Dietary restriction experiments have been proposed to provide a neat support to "Disposable Soma Theory" since a low calory diet (without starvation) induces a lifespan extension and reproductive suppression in many model organisms (Masoro and Austad, 1996), unveiling the invisible link between soma maintenance and reproduction (Shanley and Kirkwood, 2000). However, it has been shown in *Drosophila* that the life extension induced by dietary restriction does not depend on reproduction suppression, since sterile female flies strain undergo a lifespan increase when dietary restricted (Mair et al., 2004). Moreover male flies, which suffer lower reproductive costs than females, display the same changes in mortality than females when food levels are reduced (although to a lesser degree than females) (Mair et al., 2003). Studies conducted on *Caenorhabditis elegans* in the Kenyon laboratory at University of California at San Francisco, showed that complete gonads ablation does not affect lifespan, against the prediction that more energy is available for soma maintenance and longer life shoud be observed (Kenyon et al., 1993). Interestingly, however, a selective ablation of germline precursors in *C. elegans* produced a significant lifespan increase, suggesting that a *death signalling system* is controlled by the gonads (Hsin and Kenyon, 1999), but does not actually depends on reproduction.

1.3.4 Adaptive Programmed Death Theory

August Weismann (1834-1914), the great german biologist and advocate of germ-plasm theory, postulated that organisms evolve death programs because by so doing they clean up the living space (make room) and free up resources for younger generations.[9] Weismann suggested that such death program was determined by the programmed number of cell generations which cells of a given tissue undergo during their life, a concept later described as the *Hayflick limit* (Effros, 2004).

During XX century this theory was put apart since it claimed an "heretic" view of evolution based on population as the object of selection. The major point raised by George Williams (Williams, 1957) against a "program for death" theory grounds on the

> ... difficulties involved in visualizing how such a feature could be produced by natural selection

Programmed death poses and apparent evolutionary paradox since it would be theoretically disadvantageous for individuals of a lineage to inherit a program for death, i.e. reducing survival reduces individual fitness, whereas it is widely assumed that individuals of a given population evolve strategied which maximize their fitness. Recent theoretical models have raised the possibility that in conditions of limited resources, it would be advantageous, in a popoulation survival perspective, to have individuals with suboptimal individual fitness (Rauch et al., 2002). Maximized individual fitness and multiple generations overlap would cause resources overexploitation and consequent population chaotic dynamics which could lead to extinction. In this cases it would be advantageous for the adaptability of a population to

[9] reported by Gavrilov and Gavrilova (Gavrilov and Gavrilova, 2002).

evolve a mechanism of programmed death wich would reduce each generation's half-life (Weismann, 1889; Mitteldorf, 2001; Longo et al., 2005).

1.4 Aging Models

Current research on aging makes use of a wide range of model organisms, from yeasts to monkeys, through worms, flies, fishes, rodents and dogs. Here I will describe the experimetal models that, since the beginning of the nineties of the XX century, contributed to a deep understanding of some of the mechanisms concerning aging.

The yeast *Saccharomyces cerevisiae* is used as a model of replicative senescence, since after several cell divisions, late in life, yeast become enlarged and face a progressive decline in the capacity to undergo asymmetrical cell divisions (Nemoto and Finkel, 2004). Yeast have been fundamental for discovering the importance of Sir2 family members, a class of NAD-dependent histone deacetylase which maintain genome stability, as key factors for prolonging lifespan (i.e. number of clonal divisions) on a low-calorie substrate (Lin et al., 2000; Lamming et al., 2005).

The nematode *Caenorhabditis elegans* helped unveiling the molecular pathways which transduce nutritional state signals into pathways which modulate lifespan (Riddle, 1997). Studies on *C. elegans* have shown that aging can be finely regulated hormonally through the Insulin/IGF-1 pathway, a pathway later shown to influence lifespan not only in worms, but also in flies and mammals (Tatar et al., 2003). Worms that have mutation in the gene *daf-2*, which encodes a gene for an insulin/IGF-1 receptor ortholog, lived twice as long as the wildtype (Kenyon et al., 1993; Kimura et al., 1997). This lifespan extension was achieved trough the action of *daf-16*, a gene which encodes a Forkhead box transcription factor belonging to the FOXO family (Lin et al., 1997; Ogg et al., 1997). Interestingly, Dillin *et al.* reported how lifespan control by insulin/IGF-1 pathway can be dis-

sociated from growth and reproduction in *daf-2* RNAi experiments (Dillin et al., 2002), against predictions made by Antagonistic Pleriotropy Theory of aging that longer lifespan would be necessarily associated to *decreased vigor* in youth.

Heteroallelic mutant female flies for the Insulin/IGF-1 receptor are dwarf and have an extension of adult longevity of 85% compared to wild-type (Tatar et al., 2001). Mutants in *chico*, a downstream insulin receptor substrate (IRS)-like signalling protein, increase lifespan by 40% (Clancy et al., 2001; Tu et al., 2002). Similarly to worms, life extension through Insulin/IGF-1 pathway seems to be FOXO-dependent also in flies, where FOXO overexpression extends lifespan (Hwangbo et al., 2004; Giannakou et al., 2004). Interestingly, FOXO induces lifespan extension also in flies with increased basal levels of JNK (Jun-N-Terminal Kinase), which induces a protective gene expression program (Wang et al., 2005; **?**). Similar results were also found in the worm *C. elegans*, where JNK is a positive regulator of DAF-16 forkhead transcription factor and its overexpression is responsible for significantly increased longevity (Ho et al., 2005).

Mammals developed different specific receptors which differentiate insulin and IGF-1,[10] and mutant mice hetherozygous for a null mutation of the IGF-1 receptor live about 30% longer than wild-type (Holzenberger et al., 2003).[11] Moreover, mice with a tissue specific deletion of the insulin receptor in the adipose tissue live, about 18% longer than wild-type (Bluher et al., 2003). Are FOXO proteins key factors in mammals lifespan extension? The answer to this question is not yet known, but there are several indications that this could be the case (Kenyon, 2005). FOXO proteins are implicated

[10] unlike worms and flies, which share the same receptor.
[11] males live 16% longer, not significantly more than controls.

in mouse insulin and IGF-1 pathways that affect metabolism (Burgering and Kops, 2002) and, interestingly, there are evidences that mice with disrupted p66shc adaptor protein, which are 30% longer living than controls (Migliaccio et al., 1999), regulate mammalian Forkhead activity and this may lead to an increase in antioxidants such as catalase and superoxide dismutase (Nemoto and Finkel, 2002). In normal mice, under stress conditions, FOXO family is implicated in stress response (Brunet et al., 2004). As stress resistance is positively associated to longevity (Kirkwood and Austad, 2000), FOXO factors could mediate lifespan increase in mammals.

Simple model organisms, such as yeasts and worms, helped identifying several genes which regulate lifespan and aging also in other classes of organisms (Kaeberlein et al., 2002), including mammals. This functional conservation highlighted the existence of a common pathway for aging across taxa (Kenyon, 2001), against the prediction made by the "Mutation Accumulation Theory of aging" which states that aging is a phenomenon of mutational load, or, worse, the deleterious effect of recently acquired mutations that have yet to be eliminated by natural selection.

Invertebrate model organisms like yeasts, worms and flies happen to be very useful for aging research due to their small size and, most importantly, to their short lifespan and inter generation time interval. Small size and short lifespan make it possible to run several life-long trials with a reduced expense in terms of food, space and time. Although invertebrate models have provided very powerful tools for understanding general mechanisms concerning aging in all taxa, it is also true that some limitations can be found. Worms and flies are multicellular organisms in which adults have almost all post-mitotic tissues (excluding gonads), and do not share the same diseases associated with aging in organisms with actively repli-

cating adult tissues, like vertebrates. Organisms with high rates of mitosis in peripheral tissues need to have finely tuned mechanisms for preserving replication (which can lead to development of neoplastic transformations) and, at the same time, for avoiding an unnecessary cell cycle arrest or apoptosis, which would prevent efficient tissue repair. Replicative senescence, for instance, is believed to be a mechanism evolved for protecting tissues from the development of lethal neoplastic transformations (Campisi, 2005b). Recent findings show that pre-malignant non lethal cancers express senescence markers, whereas malignant tumors do not (Collado et al., 2005; Chen et al., 2005), suggesting an adaptive mechanism aimed at dampening the onset of cancer by cellular senescence (Campisi, 2005c).

Thus, it would be useful to discover, study and develop new vertebrate aging models without the practical inconvenience of the long lifespan of current rodent models.[12] In addition, it would be important to identify vertebrate model with naturally evolved short lifespan phenotypes, i.e. not produced by artificial selection or genetic manipolations aimed at "accelerating aging" or at reducing lifespan (Miller, 2004). Indeed, the risk with artificially produced short-living aging models is to study, instead of a real aging phenotype, an increased frailty (and consequent increased death risk) due to early disease onset.

[12]laboratory mice stocks live about 2.5 years (Holzenberger et al., 2003), whereas wild derived ones live much longer (Miller et al., 2002).

1.5 Aging Markers and Age-Associated traits

Age-related functional and structural dysfunctions occurring with senescence are often associated with the onset of typical histological markers, commonly termed *Aging Markers*. The association of age-dependent histological *aging markers*, behavioral impairment, and exponential increase of death rate, is a typical array of markers characterizing senescence. In this thesis I used a set of histological aging markers useful to highlight periferal tissues and central nervous system aging. Here follows a list of aging markers used in this thesis.

- *Lipofuscin*, or fluorescent age pigment, is presented as yellowish-brown pigment granules containing residues of lysosomal digestion. It progressively accumulates in muscle fibers, liver, kidney, adrenals and neural cells. *Lipofuscin* can be detected by Lysosomal stains, Ferric ferricyanide reduction test and autofluorescence. Lipofuscin is indeed a conglomerate of lipids, metals, organic molecules likely produced within secondary lysosomes by the interplay between reactive oxygen species production by the mitochondria and autophagocytotic degradation within secondary lysosomes (Brunk et al., 1992).

- *Senescent Associated β Galactosidase* has been first used as a marker of cellular senescence in human fibroblasts which lose the capacity to replicate *in vitro* and *in vivo* (Dimri et al., 1995). It is histochemically detectable as *X-gal* staining at pH 6 and was also found *in vivo* in senescent zebrafish fibroblasts (Kishi et al., 2003), in oncogene-induced mice (Collado et al., 2005), and in human benign tumors of melanocytes (Michaloglu et al., 2005). It reflects an increase of the

acid lysosomal β galactosidase, apparently associated with an increase in lysosomal content in senescent cells (Kurz et al., 2000).

- *Amyloid Plaques* deposition in the brain is a classical marker associated to age-associated neurological disorders, the most known being *Alzheimer's Disease*. Spawning senescent salmon is the first wild senescent vertebrate to show brain accumulation of β-Amyloid plaques (Maldonado et al., 2000). The accumulation of β-Amyloid is associated with abnormal cleavage of APP (Amyloid Precursor Protein) by specific enzymes termed β and γ secretases. A-β amyloid fragments, when aggregated, induce the formation of senile plaques. Amyloid plaques can be detected by specific antibodies or through the classical Congo Red coloration.

- *Tau Protein Neurofibrillary Tangles* (NFT) form up on aggregation of a hyperphosphorylated microtubule-associated tau-protein. These intraneural aggregates are insoluble, dysfunctional structures and are typical markers of *Alzheimer's Disease*. Tau proteins are very conserved and their presence was also reported for zebrafish and goldfish (Tomasiewicz and Wood, 1999), although pathological tangles formation is not reported.

- *Fluoro-Jade B* is a specific marker for neuronal degeneration (both apoptosis and necrosis) that was first developed in rodents (Schmued and Hopkins, 2000) but which is also successful in fishes (Bettini et al., 2005).

The occurrence of age-related histological markers is associated with behavioral impairments (see later) which often occurr with aging, but a causal

connection between these two classes of age-related traits is not easy to be found.

Aging is often associated to a decrease in spontaneous locomotion and spacial exploration as a result of degeneration in the central and/or peripheral nervous systems as well as in musculoskeletal system. Associated with locomotor decline is a decreased muscle mass (Anderson, 2003), effect due to a loss in the number of muscle fibers (Lexell, 1993).

In humans, declining memory and cognitive functions are considered age-associated traits (Craik and Salthouse, 1999). Cognitive decline is typically associated with aging brain in many animal models, which invaluably contribute to the understanding of the genetic and physiology of such phenomenon. Studies on *Drosophila* indicate that mutant flies for the *amnesiac* gene show, at young ages, the same memory impairment of aged flies, which completely lose *Middle Term Memory*, a labile form of memory which lasts up to 7 hours after training. This kind of memory is very efficient in young flies, but decays in older ones (Tamura et al., 2003; Horiuchi and Saitoe, 2005). Interestingly, the *amnesiac* gene is homologous to vertebrate's pituitary adenylyl cyclase-activating peptide (PACAP) and growth hormone-releasing hormone (GHRH), and mutant flies for *amnesiac* live longer than wildtype.[13] Rodents undergo memory loss (spatial memory) in elderly and this is attributed to general mitochondrial failure and oxidative damage (Liu et al., 2002), expecially in the hippocampus, striatum and cerebellum. Studies which integrate histological age-related markers with functional markers, such as behavioral impairments, can help understanding the causal correlations between these different classes of age-related traits.

[13]Horiuchi J., personal communication.

1.6 Environmental treatments affecting lifespan

The rate of aging and maximum lifespan can be modulated by environmental factors like diet, environmental temperature, stressors and drugs, i.e. aging and lifespan are also under epigenetic control.

1.6.1 Food Restriction

Diet significantly influences lifespan in diverse model organisms, including yeast, rotifers, spiders, worms, fish, flies, rodents and, possibly, non-human primates (Weindruch and Walford, 1988; Lane et al., 2001). Dietary restricted mice displayed reduced cancer incidence (Weindruch and Walford, 1982) and long-term calory restriction reduces the risk for atherosclerosis in humans (Fontanta et al., 2004).

The molecular pathways which mediate the effects of food restriction is a highly debated subject. In addition there are evidences that dietary restriction does not act on the same pathways in all models. With a reduced glucose substrate, yeasts undergo metabolic shift from fermentation to respiration, increasing their replicative lifespan (Lin et al., 2002). An analog increase in respiration is also observed in food restricted worms (Houthoofd et al., 2002) and mice (Nisoli et al., 2005). The way in which an increased respiration induces a lifespan extension in yeasts is not yet clear, but it is known that it requires the action of the histone deacetylase Sir2[14] for inducing lifespan extension. Sir2's ability to extend lifespan is conserved during evolution and its overexpression increases lifespan in worms and flies (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). Sir2 mam-

[14] although there exists a Sir2 independent Dietary Restriction pathway in yeasts (Kaeberlein et al., 2004).

malian ortholog SirT1 is involved in controlling FOXO dependent activation of stress response genes (Brunet et al., 2004) and is induced by Dietary Restriction in rat (Cohen et al., 2004) and mice (Nisoli et al., 2005).

Insulin/IGF-1 pathway integrity is fundamental for life extension due to dietary restriction in flies (Clancy et al., 2002) but not in worms, where life extension is induced also with a reduced DAF-16/FOXO signaling (Houthoofd et al., 2003). In mammals, it is not yet known whether insulin and IGF-1 pathways play a role in life extension due to dietary restriction (Kenyon, 2005). Acute starvation induces up-regulation of pancreatic UCP-2 protein through the action of SirT1, which eventually leads to insulin plasma levels decrease (Bordone et al., 2006). Moreover, dietary restriction decreases plasma levels of IGF-1 but increases tissue levels of IGF-1 and type 1 IGF-1 receptors (Sonntag et al., 1999), and long-lived dwarf mice also display low levels of IGF-1 (Coschignano et al., 2000). Interestingly, Ames dwarf mice subjected to dietary restriction live longer than control fed ones (Bartke et al., 2001b), possibly suggesting an ucorrelation between the mechanisms linking dietary restriction and IGF-1-pathway to lifespan increase.

The TOR (Target of Rapamycin) signaling pathway, regulates cell growth and proliferation, is activated in response to nutrients, and its downregulation has been suggested to be responsible for dietary restriction dependent life extension. In fact, its decreased activity extends lifespan both in worms and flies (Jia et al., 2004; Kapahi et al., 2004) and dietary restriction does not further extends lifespan in mutant flies.

1.6.2 Low Temperature

Environmental temperature is importantly connected with lifepan and aging rate in poikilotherms as different as worms, flies and fishes, where animals kept at lower temperature live longer (Liu and Walford, 1975; Van Voorhies and Ward, 1999; Mair et al., 2005) and generally have lower metabolic rates (Speakman, 2005a). Endotherms raise their metabolic rates in response to lowered ambient temperatures to keep their body temperature constant. Since smaller bodies are more likely than larger ones to get close to their lower critical temperature, it is observed that, within a given mammalian species, smaller subjects need to produce heat by mitochondrial uncoupling *via* UCP-1 (Cannon and Nedergaard, 2004) and, interestingly, they also live longer (Speakman, 2005a). Studies on zebrafish showed that an ambient temperature reduction induced significant transcripts upregulation in two gene ontology functional groups: "oxygen and reactive oxygen species metabolism" and "response to oxidative stress" (Malek et al., 2004; Gracey et al., 2004). It is therefore likely that the increased lifespan observed in animals kept at lower temperatures is in part the result of the low-temperature-dependent enhanced metabolic response against oxidative damage.

1.6.3 Oxidative Stress

Studies on *C. elegans* and *Drosophila* showed that low level of oxidative stress induce subsequent beneficial effects, a phenomenon known as "hormesis". Transient heat shock extend lifespan in flies and worms (Apfeld et al., 2004; Hercus et al., 2003; Lithgow et al., 1995). Since overexpression of heat-shock factor HSF-1 extends lifespan of *C. elegans* (Morley and Morimoto,

2004), it is likely that stress activates HSF-1, eventually increasing lifespan by activating downstream life-extending genes. Moreover, the activation of the stress-response Jun-kinase (JNK) pathway in *Drosophila* extends lifespan by 80% (Wang et al., 2003), requires Foxo and antagonizes systemically insulin/IGF-1 signaling by repressing insulin/IGF-1 signaling ligand expression in neuroendocrine cells (Wang et al., 2005).

1.6.4 Resveratrol

Resveratrol, a phytoalexin found in grapes and red wine (Granados-Soto, 2003), increases longevity in *S. cerevisiae*, *C. elegans* and *Drosophila* (Howitz et al., 2003; Wood et al., 2004). *Resveratrol* has been reported to have anticancer and anti-inflammatory action in vitro (Jang et al., 1997; Manna et al., 2000), neuro-protective action in vitro (Araki et al., 2004; Parker et al., 2005; Wang et al., 2002; Wang et al., 2004; Han et al., 2004) and in vivo (Wang et al., 2004). The mechanisms of action of *resveratrol* are multiple (Granados-Soto, 2003). Resveratrol inhibits mitochondrial ATPase in mammals (Zheng and Ramirez, 2000; Gledhill and Walker, 2005) and was reported to activate the NAD-dependent hystone deacetylase sirtuins in nematodes, flies and rodents (Wood et al., 2004; Araki et al., 2004). Over-expression of the mammalian homologue of Sir2, SIRT1, has reported to be neuro-protective in a variety of models, and this effect is mimicked by *resveratrol* (Araki et al., 2004; Parker et al., 2005; Han et al., 2004). Moreover, dietary restriction causes over-expression of SIRT1 (Cohen et al., 2004) and its effects on lifespan are not additive to those of *resveratrol* in *Drosophila* (Wood et al., 2004). These data raise the possibility that *resver-*

atrol mimics dietary restriction by decreasing the aging rate through the activation of SIRT1. However, more recent results have shown that *resveratrol* increases Sir2/SIRT1 activity only if the substrate is conjugated to a non-physiological fluorescent moiety (Kaeberlein et al., 2005; Borra et al., 2005), suggesting that the association between dietary restriction and resveratrol's action needs further investigation (see Discussion).

Resveratrol has shown neuroprotective activity in a variety of paradigms both *in vivo* and *in vitro* (Araki et al., 2004; Parker et al., 2005; Wang et al., 2002; Wang et al., 2004; Han et al., 2004), raising the intriguing possibility that it would regulate lifespan through its action on the brain. Invertebrate studies have revealed the importance of the nervous system in regulating lifespan, as neuronal-specific gene manipulations and neuroprotective drugs can both change the longevity of worms and flies (Evason et al., 2005; Parkes et al., 1998; Apfeld and Kenyon, 1999; Wolkow et al., 2000). In mammals, the nervous system is able to regulate lifespan, as an underdeveloped anterior pituitary is associated to a significant longer lifespan in Snell and Ames dwarf mice (Bartke et al., 2001a).

Whether resveratrol positive effect on lifespan is dependent on its action on the nervous system is to be demonstrated, moreover, further studies are needed to understand whether resveratrol is also able to increase vertebrates lifespan and whether its effect on lifespan is dependent on the same pathways involved in the response to dietary restriction.

1.7 *Nothobranchius furzeri* ecology and natural history

Fishes of the genus *Nothobranchius* are annual Oviparous Cyprinodontiforms belonging to the Aplocheilidae family (Murphy and Collier, 1997). As annual aplocheiloids, they reside in temporary pools which regularly dry up. Therefore survival in dry season depends on the capacity of the embryos to undergo embryonic development and one or more diapauses (from diapause I to diapause III) in the dry mud. Diapauses enable embryos to survive the dry season until the next rainfalls rewet the habitat (Murphy and Collier, 1997) and give the larvae the chance to hatch.

Nothobranchius furzeri, like all *Notobranchius* species, have a marked sexual dimorphism and dichromatism, as shown in Fig.1.1

Figure 1.1: Male (above) and female (below) adult *Nothobranchius furzeri*. Photo of the female fish by A. Dorn ©.

Male adults measure in average about 5 cm of total-length and excep-

tionally reach 5 cm, whereas females rarely measure more than 3.5 cm.

Nothobranchius furzeri, also called "Turquoise killifish", was discovered in the sixties in modern Zimbabwe, in the Sazale Pan of Gonarezhou Game Reserve by Furzer and Warne (Jubb, 1971). The Reserve of Gonarezhou lies on the lowveldt of southern Zimbabwe, between 300-600 m above sea level, adjacent to the most southern part of the border with Mozambique and very close to the Limpopo river on the south, natural border between Zimbabwe and South Africa. *Gonarezhou* National Park is delimited by the Runde River (draining to Save River) on the North side and Mwenezi River (draining to Limpopo River) on the South side. Both Runde and Mwenedzi River run from W/NW to E/SE right into Mozambique. Within *Gonarezhou*, also the Guluene and Chefu River -from Zimbabwe- converge into Mozambique, contributing to the Changane River which evolves into a series of swamps, pans and lakes within the province of Gaza. In the *Gonarezhou* area, the rains fall seasonally and the wet season takes place usually between October and February with 250-400 mm of rain per year, [15] all concentrated in this brief time frame, with some exceptional years without rains.

Although not yet thoroughly clear, the home range of *N. furzeri* is roughly delimited at north by the Save River, at south by the Limpopo River,[16] at west by the *Gonarezhou* and at east it does not extend over an ideal line distant 100 km from the humid ocean coastline. The geographical distribution of *N. furzeri* in part overlaps with that of *Nothobranchius orthonotus* (also found in Sazale pan by Furzer and Warne in the late sixties)

[15] very dry indeed! A mediterranean city like Bari receives in average 700 mm rain per year...

[16] B. Watters found *N. furzeri* populations beyond the south-west Limpopo border (Watters, 2004).

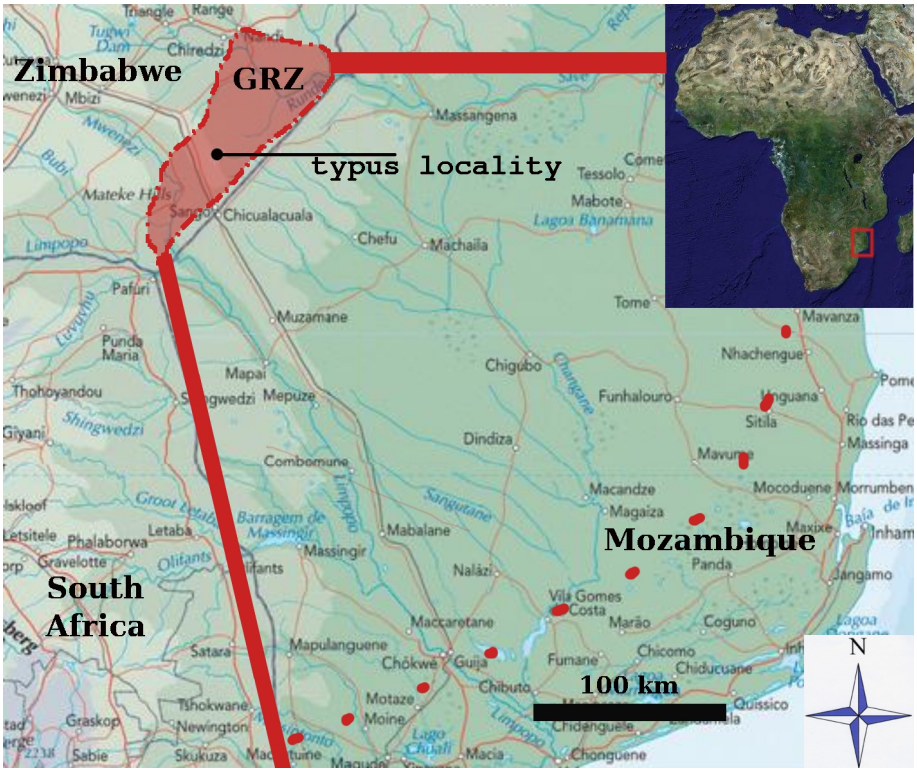

Figure 1.2: Geographical distribution of *Nothobranchius furzeri*. In the small upper-right *insert* it is represented the area realtive to the bigger map within the Southern African sub-continent. *Larger panel*: in pink is shaded the *Gonarezhou* (GRZ) National Park, which is the place where the species was found in 1968-69 and the holotype was captured. The typus locality is evidenced by a black dot. The thickest grey line is the Country border. The eastern GRZ side lies on the border between Zimbabwe and Mozambique. Map obtained by the URL: www.multimap.com. Geographical distribution of *N. furzeri* corresponds to the area delimited by the pink lines and dots.

and *Nothobranchius rachovii*, species with much wider distribution, ranging form Kruger National Park in South Africa up to the River Zambesi.

A typical trait of this species is the color pattern of the male-tail, which

presents a yellow (proximal) and black (distal) vertical stripe (Jubb, 1971). Mr. Trevor Wood collected in 1999 a *Nothobranchius sp.* population with red-tail males in the region of Chockwe, in Mozambique, and described it as a possible sub-population of *N. furzeri*. GRZ inbred line is believed to descend from two pairs of *N. furzeri* reproduced by Walter Foersch in Germany.

In this thesis I study the GRZ inbred line and 4 wild-derived populations which were collected in two different collection trips which took place in Mozambique in 2004 (see next section and *Results* chapter).

1.8 Thesis Design and Scope

In this Thesis, I describe age-associated traits in *Nothobranchius furzeri*, a new vertebrate model for the study of aging. In particular, I characterize aging traits and markers in captive strains of *N. furzeri* used as experimental subjects and subjected to different experimental treatments: different water temperatures and fed with 3 different doses of *resveratrol* in the food. I moreover characterize locomotor and cognitive age-dependent decline as well as histological markers of senescence in all these groups. In *section 3.5* of *Results* chapter, I compare survival curves and behavioral measures of the inbred captive strain with that of wild derived populations, obtained during a collection trip in Mozambique to which I took part in Spring 2004. Details on the collection trip will be given, including the GPS coordinates of the localities where all the popoulations where found.

1.8.1 Results chapter structure

In the first section of *Results* chapter, I analyze the aging-associated traits in the inbred *N. furzeri* strain, called GRZ strain (from *Gonarezhou*). The characterization of the aging phenotype consists in the study of survival (survival curves) in both sexes and death rate curve computation, a widely used method considered to provide an indirect measure of individual aging-rate through the increased population death risk (Gavrilov and Gavrilova, 2001). For scoring markers of senescence of 9 weeks old fishes, I show immunohistochemical analysis (see Materials and Methods chapter for details) of brain (*optic tectum*) lipofuscin, Amyloid Aβ plaques, tau-protein tangles; liver lipofuscin and fibroblasts SA β Galactosidase. I quantify locomotor and cognitive decline as integrate functional measures of senescence (see

Materials and Methods for details on such measures).

In the second section of *Results* chapter, I analyze the effects of three different water temperatures on age-associated traits in the GRZ strain. In particular, I compare the survival curves of the three groups and the death rate curve in the two most numerous groups. I also score body size as well as locomotor and cognitive decline.

In the third section, I score aging associated traits in three groups of fishes fed with different doses of *resveratrol* in the food. In these groups, I compare fluoro-jade marker for neurofibrillary degeneration, survival curves and death rate curves, locomotion and cognitive decline.

In the last sections of *Results* chapter, I compare the survival curves and the death rate curves of the GRZ strain with that of wild captured populations and I also quantify survival in hybrid lines generated by crossing short living inbred fish with long-lived wild-derived fish. Moreover I quantify significant differences in learning abilities and open field tests as measure of general motility in different strains, discovering interesting differences which would deserve further analysis.

The scope of this thesis is to show that the annual killifish *Nothobranchius furzeri* is a powerful aging model. All aging model organisms with a lifespan comparable to that of *N. furzeri* are invertebrates (e.g. *Drosophila*) and do not share some features which are typical of vertebrate's aging, like cellular senescence and neoplastic transformations. Invertebrate models like *C. elegans* and *Drosophila melanogaster* have all post-mitotic adult tissues, excluding gonads. The principal features which make *N. furzeri* a powerful model for the study of aging are: short lifespan, natural aging phenotype, genetic proximity to species for which genetic resources

have largely been developed, like stickleback, fugu, tetraodon, medaka and also zebrafish (see *Discussion* chapter), but it is also worth mentioning that, in general, fishes of the genus *Notobranchius* have no special housing requirements, are easy to reproduce and easy to be exchanged between labs. *Nothobranchius* eggs survive to desiccation and are frequently posted by mail among hobbyists. Noteworthy, *N. furzeri* are ideal models for artificial selection experiments for their short inter-generation time interval,[17] which is about 7-8 weeks,[18] and offer a precious chance for researchers interested in studying, in the same model, topics like the genetics of longevity but also speciation and molecular evolution.

[17] the time which goes from parent's first day after disclosure to offspring's first day after disclosure.

[18] i.e. in one year, more than 6 generations can be produced by any two progenitors.

Chapter 2

Materials and Methods

2.1 Housing Conditions

Fish care and housing followed a specific protocol depending on the age of the fish and on the chosen experimental conditions. In standard conditions adult fishes were housed in 40 l tanks at 25 °C, with an "air supplied" water filtration and low water flow, since strong water flow can be lethal for this genus, as already reported for *Nothobranchius rachovii* (Herrera and Jagadeeswaran, 2004; Genade et al., 2005). We performed 50% water change every week and dayly removed dead fishes from the tanks, providing a 10% water change for each dead fish, in order to avoid water pollution due to a raise in ammonium. It is also worth mentioning that keeping stable water parameters is not a major issue in *Nothobranchius* care (Watters, 1998; Genade et al., 2005), as they live in nature in stagnant pools and can survive to poor water quality and a wide range of temperatures. We chose to keep them in hard, alkaline water, as suggested by AKA guidelines (American Killifish Assiciation) (Markis and Langton, 1990). Food was provided

twice a day, and we chose to use the commercially available frozen bloodworm larvae (*Chironomus sp.*) provided by a local source, a food rich in proteins and fatty acids. The amount of food released in tanks equaled 50mg(food)/g(fish)/day (Valenzano et al., 2006). Fishes were fed manually and, within 2 hours, not eaten food was removed from the tank in order to avoid water contamination. We exposed fishes to light cycles of 12 hours (i.e. 12 hours of light and 12 hours of dark). Fish density was 20 fishes per tank from the 4th week of life. Adult fishes were let spawning on a river-shore sand substrate, which was weekly sieved and put back in the bottom of the tanks. Sieving allowed us to remove eggs form the sand, which were thereafter placed on a moist peat moss substrate for dry-incubation. The peat substrate filled a 10cm diameter petry dish and eggs were gently placed on the top of it. This allowed us to dayly check for dead or unfertilized embyos which were removed by the peat in order to avoid fungal infections to contaminate the whole dish. On average, about one fourth of all eggs become infected, appearing pale and developing the caracteristic white filamentous fungal coat. Healthy eggs appear amber in color right after being put on the peat. Every single petri dish was closed with parafilm to avoid excessive desiccation which can kill all the embryos. The peat we used was non-chemically treated and, before using it for incubation, we boiled it for 3 hours and kept it in a sterile box. "Eyed up" embryos, i.e. embryos showing a well defined golden eye-ring, which is considered as an indicator of completed development (Scheel, 1975), were put in a 2l tank, with peat extract, oxygen tablets to avoid *belly sliders* (fries which did not succeed in filling with air the gas bladder), peat extract and water at about 18 °C, in order to facilitate the hatching of the fries.

Fries were grown in 20l tanks at 25 °C and were dayly fed with nauplii

of *Artemia salina*, a small crustacean commonly used for raising commercial fishes until the fourth week of life, when they were moved to 40L tanks. Since the second week of life fries were fed with poly-unsaturated fatty acids (PUFA) enriched *Artemia salina*, which provided a richer nutrient support and, from the third week of life, fries started to receive also chopped bloodworms.

2.1.1 Preparation of food pellets containing resveratrol

For 120 mg/food pellet: 1.2 mg/μl resveratrol stock was prepared in 5% ethanol and stored at 4C in the dark. Frozen *Chironomus* larvae were thawed, left to drip dry, and aliquoted into portions of one feeding for 10 adult fishes (1 g). 100 μl of the 1.2 mg/μl stock was added to each *Chironomus* aliquot, which was left at 4 °C for 1-2 hr to soak. 5% gelatine was added to the *Chironomus*/resveratrol aliquot, mixed, frozen, and stored at - 22 °C until use. For feeding, the frozen gelatine/*Chironomus* cube was thawed in water and fed to the fishes. All uneaten food was removed. Fishes received 2 feedings per day. Control-fed fishes were fed with the same kind of food lacking resveratrol in the stock solution.

2.2 Methods for Scoring Aging and Longevity

2.2.1 Survival assays

Fishes started to be scored for survival assays since the fourth week of life, when they are considered sexually mature (Valdesalici and Cellerino, 2003; Genade et al., 2005; Valenzano et al., 2006) and are moved to 40l tanks with a density of 20 fishes per tank. Fishes were counted every week, and every day, we scored dead fishes. Mortality in fishes younger than 4 weeks usually occurred in the very first days after hatching.

For computing differences among different treatments, we used the commercially available ®Graph Pad and ®Origin softwares. Since we scored both alive fishes (on a weekly basis) and dead fishes (on a dayly basis), we were able to assess any incongruence among these two measures as *censored*, i.e. it can happen that starting with n fishes, after m weeks, a given amount z of fishes were removed from the tank for histology. In this case we have, at week $m-1, n$ fishes and, at week $m, n-z$ fishes, without really having any death due to increased morbidity. One can remove z fishes from the total number of fishes or, better, consider them as *censored* at week $n-1$. This measure allows to account for all fishes, without missing those which are removed by the tank for reasons different from natural death. In other situations, the sum of all dead fishes did not equal (was lower than) the initial number of fishes. This can be due to unseen deterioration of dead fish bodies in the tank (which were not scored as dead), cannibalism (although very rare among same-age *N. furzeri* but frequently observed in the first weeks of life in *N. orthonotus* var. *kuntahe*), jumping out of the tank, etc ... In all these cases, the missing fishes were considered as *censored*.

2.2.2 Histology and Immunohistochemistry

Fish histology followed a standard protocol for all tissues. Chosen fishes were euthanized with MS-222 and crushed ice for 5 min before dissection. Tissues were immersion fixed, cryoprotected, included in tissue-tex, and sliced (18 μm of thickness) by a cryostate. Fluorescence analysis were performed by the software ®Metamorph. All assays followed the specified protocols indicated on the information sheet provided by the Company from which we purchased the required reagents.

2.2.3 Behavioral Assays

For scoring age-dependent behavioral decay in *N. furzeri*, I used three different behavioral assays: an assay designed for scoring *Spontaneous Locomotion* in a social condition, an assay for scoring individual fish locomotor activity in a *Open Field*-like condition and the last for scoring learning an *Active Avoidance*. A last original behavioral assay is also introduced in Appendix, designed for scoring *Passive Avoidance*, but no data obtained with this last task is presents in this thesis.

Spontaneous Locomotion

Starting at the fourth week of life, at every mesure session, 10 fishes were left in their home tank with a digital video camera put in front of the long side of the tank. One-hour tapes with 1frame/s speed were scored in standard conditions of light and with a white background placed on the other long side of the tank in order to increase the visual contrast of the fishes over the background. From one hour recordings, I randomly chose 10 starting points (a frame) and, for each of these points, I proceeded as follows: I chose

a given value of image thresholding for all the experimental set (i.e. for all visual analysis concerning one experiment, usually with 16 bit images the threshold values were 66-100) and I performed an image subtraction between two frames distant 5 seconds, i.e. $Frame10 - Frame5$ and so on. In this way, I obtained a map of displacement between two frames distant 5 seconds one from the other. I considered a fish to be *significantly moving* when it was displaced from its position in the previous frame for more than half of its body-length. A fish was given a value of 1 when it was scored as moving and 0 when not moving. The average was computed for 10 fishes at every starting point (10 starting point for every week measure). In this way, I could score *Spontaneous Locomotion* in a age-dependent fashion. This measure provides a good approximation of natural/spontaneous motility for fishes of each age-class. The image analysis were performed with the free software ®Image J, available at the URL: http://rsb.info.nih.gov/ij/download.html.

Open-field like assay

Single fishes were scored for locomotor activity in a 20l test-tank with the same temperature of the home tank. Video recordings were performed with a digital video-camera from above and water level was kept very low in order to minimize the displacement on the z axis which would not be scored by the camera while recording from above (Fig 2.1). Fishes were let to habituate for 30 min within the tank before the 10 min recordings started. The image analysis were performed by the Software *Ethovision* (Noldus^{TM}, the Netherland) with which I computed mean, maximum velocity and percentage time spent moving for every single fish belonging to any experimental group. For every groups means and standard deviations were computed.

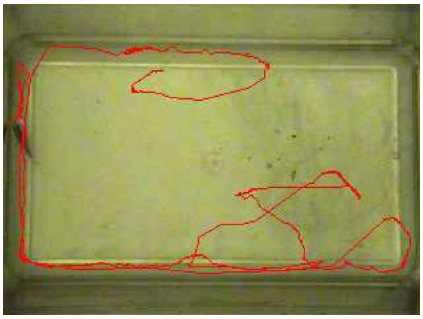

Figure 2.1: Snapshot from the *Open Field*-like test. In red is drawn the path tracked by a single fish in a sample file of 2 min. Track is drawn by the Software *Ethovision* (Noldus^{TM}, the Netherland).

Active Avoidance Task

I modified a learning taks developed for gold fish and zebrafish (Pradel et al., 1999). I engineered a shuttlebox (Fig 2.2A) consisting in a tank (38x23x18 cm) divided in two by a hurdle with a rectangular hole (3x3 cm). The two compartments were wedged-shaped to funnel the fish through the hurdle. The tank was filled with water from the housing tank and the fish was left to acclimate for 15 minutes before starting thetest. Then the conditioned stimulus (red light) was delivered in the compartment where the fish was present and it was followed by an adversive stimulus (a plastic stick whirling in the compartment). The fish always responded to the disturbance by moving to the other compartment. The aim of the test was to detect the acquisition of a strategy to escape from the adversive stimulus by crossing the hurdle upon presentation of the conditioned stimulus. The conditioned stimulus lasted for 30s. If the fish did not move to the other compartment after 15s, the adversive stimulus was delivered for 15s. The fish moved to the other compartment, resting for 30s and then the cycle was repeated. If the fish crossed the hurdle within 15s (i.e. before the onset of the red light

conditioned stimulus), the trial was scored as "success", otherwise it was scored as "failure" (Fig 2.2B). A complete session consisted of 50 consecutive trials.

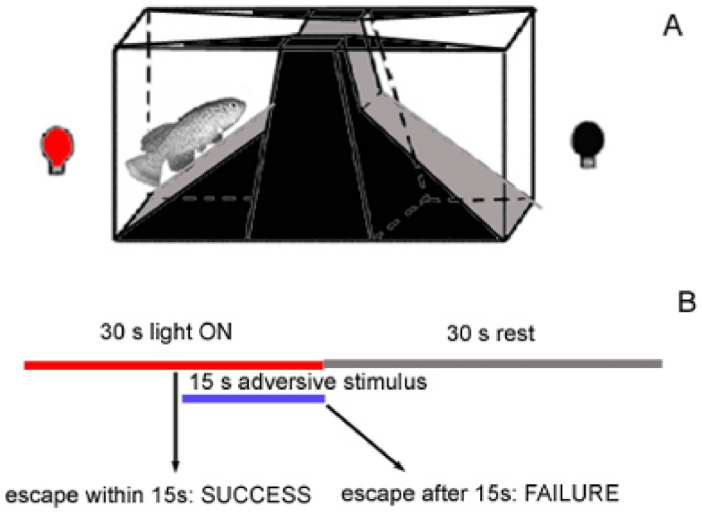

Figure 2.2: Scheme of the system used for scoring the *Active Avoidance* learning task. (A) Shematic representation of the shuttlebox used for training and testing. (B) Temporal structure of the test.

Two indexes were scored for assessing learning in each experimental group. The first measure was *Performance Index*, ad was used for visualizing the evolution of the performance in function of the trial. Succcessful trials were scored as 1, failures with 0. For each trial, I computed the average score as follows: for example, for trial 1 in n fishes, we have that Average Score is $AS_1 = \frac{1}{n}\sum_{i=1}^{n} P(i)$, where $P(i)$ is 0 or 1. The performance index *(PI)* evolution was then obtained averaging 10 consecutive AS_i from trial 10 to 50 and plotting each score as follows: $PI_{10-50} = \overline{AS}_{1-10}, \overline{AS}_{2-11}, ..., \overline{AS}_{41-50}$.

I considered the *chance level* against which to compare the obtained measured as the average score of the first trial (i.e. before the occurrence of the conditioning) of all tested fishes.

I also measured another index, called *Top Score Index* (TSI) , which measured the mean of the highest scores reached by individuals of one group during 50 trials. Compared to the *Performance Index*, *Top Score Index* provides an absolute measure of ability to succeed in the task, independently from the trial. TSI is computed as the mean, for all the individuals in a experimental group, of $max(\frac{1}{10}(\sum_{i=1}^{10} Pi, \sum_{i=2}^{11} Pi, ..., \sum_{i=41}^{50} Pi))$.

Chapter 3

Results

3.1 Aging in GRZ inbred *Nothobranchius furzeri* strain

3.1.1 Survival and Growth

A representative survival curve of inbred *Nothobranchius furzeri* GRZ strain is shown in Fig 3.1A. The graph represents the percentage of fishes that survive from the fourth week of life until the death of the last animal. At four weeks fishes are sexual mature as males show typical adult coloration (Genade et al., 2005) and females start to lay fertilized eggs in the chosen substrate (see *Method* chapter). *N. furzeri* survival curve is superimposed with that of a wild derived *Drosophila melanogaster* sample curve. The two curves show similar maximum lifespans,[19] but slightly different median lifespan, [20] indicating that while flies have a regular death trend of decrease in survival, without any abrupt change in the slope, *N. furzeri* undergo a

[19] measured as the age of the tenth percentile of survived individuals
[20] measured as the age-value corresponding to 50% of survived individuals

sudden fall in survival after the 6th week of life.

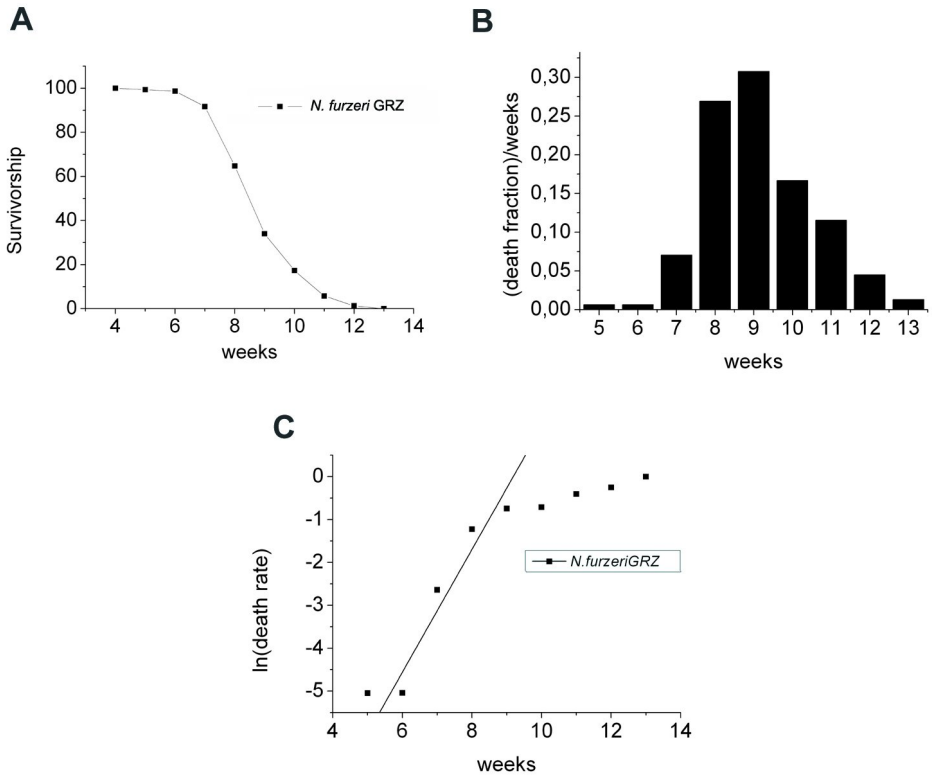

Figure 3.1: Survivorship in captive inbred GRZ (Gonarezhou strain) *Nothobranchius furzeri* kept at 25 °C. (A) Time-dependent survivorship of 156 *N. furzeri*. (B) Weekly death in *N. furzeri* expressed as the fraction of total number of individuals which die week by week. (C) Death rate curve in *N. furzeri*. The linear interpolation is referred to the linear phase of death rate increase.

The frequency histogram of mortality shown in Fig 3.1B shows that during week 8 and 9, half of all the fishes die.

The natural logarithm of death rate is plotted in Fig 3.1C in order

to visualize the exponential increase in death rate occurring with age, as expected in aging organism by the Gompertz law of mortality (Gavrilov and Gavrilova, 2001; Finch et al., 1990).[21] A linear interpolation is performed between death rate values form week 6 to week 9, corresponding to the median lifepan value for *N. furzeri*. The linear phase of death increase demonstrates an interesting pattern of early accelerated mortality, possibly linked to a aging. During the linear phase of death rate increase, the time needed for doubling mortality rate (DMR) is 0.483,[22] which means that in less than half a week the mortality rate doubles. This same index is 14.08 for the mouse and 15.64 for the rat (Finch et al., 1990), i.e. if we assume DMR to be a measure of aging, *N. furzeri* ages about 30 times faster than laboratory mice and rats.

The death rate curve present in Fig 3.1C shows a prolonged late life mortality deceleration, a trend often associated to death rate curves of aging organisms (Rose et al., 2002; Gavrilov and Gavrilova, 2003).

A comparison between survivorship and growth rate in two species of the genus *Nothobranchius* is representative of the high variability in life expectancy present in annual killifish. Inbred GRZ *N. furzeri* and wild derived *N. orthonotus* var. *kunthae* strongly differ for life expectancy in the same culture conditions, as the second one lives at least 3 times longer than the first one (Fig 3.2 A–B). Interestingly, measuring growth rates in this two species, it appears that *N. furzeri* reaches its plateau size significantly

[21] $M(t) = Ae^{\alpha t}$; $M(t)$ is mortality rate at time t, A is aggregate environmental danger and α is the rate constant for age-related increase in mortality.

[22] time needed for doubling mortality rate (DMR) is measured as follows $DMR = \frac{\ln 2}{\alpha}$, where α is the rate constant for age-related increase in mortality (Finch et al., 1990).

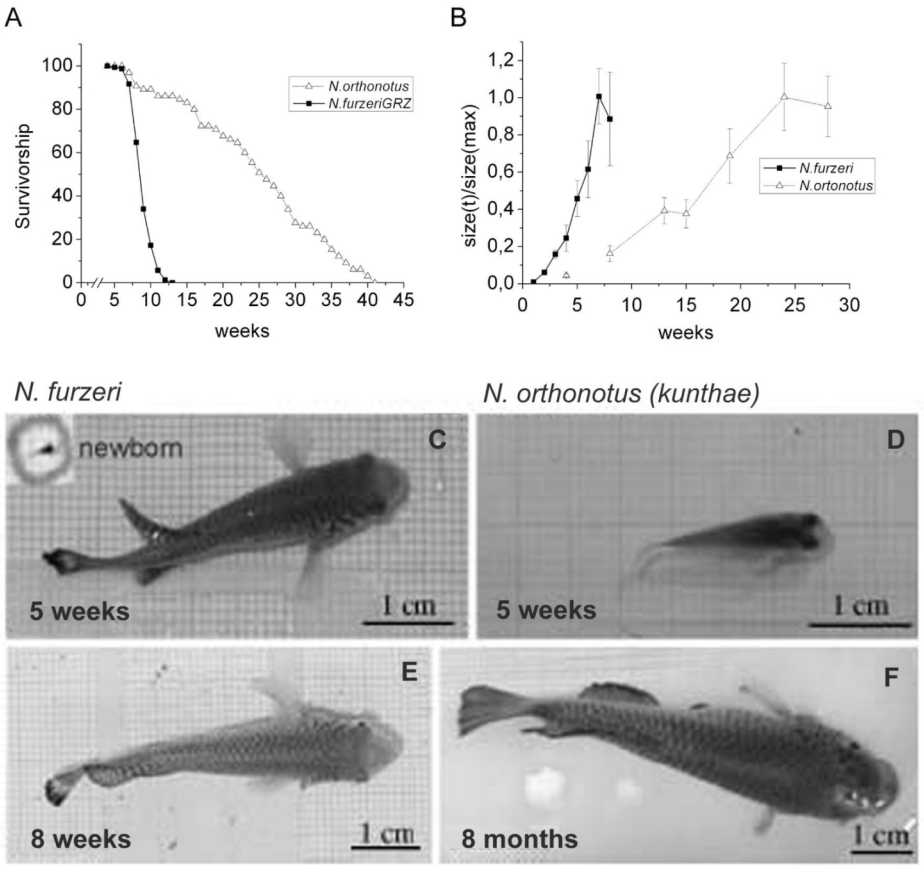

Figure 3.2: Survivorship and growth in two annual killifish species grown in separate tanks with the same culture conditions, exposed to the same light regime and water temperature of 25 °C. (A) Survival in 70 *Nothobranchius furzeri* and 65 *Nothobranchius orthonotus* var. *kunthae*. (B) Growth computed on volumes estimated by pictures through the ellipsoid formula $V = \frac{2}{3}\pi w^2 L$; w is fish maximum width and L is standard length. (C–D) Comparison of size at 5 weeks of age. (E–F) Comparison of size at 8 weeks of age (the scale used in the 5 weeks and 8 weeks comparisons is different).

before *N. orthonotus* var. *kunthae*, which grows much slower but eventually

reaches a larger sizes (Fig 3.2 C–F).

The rapid growth rate of *N. furzeri* is also coupled to an accelerated sexual maturation, which can be achieved in 4 weeks (in Fig 3.2C is shown a 5 weeks old sexually mature male *N. furzeri*), when males show distinctive yellow and black tail flags and females lay fertilized eggs. In fishes of the species *N. orthonotus* var. *kunthae* sexual maturation is reached later, around week 7.

These differences in survorhip, growth rate and sexual maturation in this two species, are probabily linked to a selection for rapid reproduction in *N. furzeri*, which live in ephemeral environments and which can possibly beneficiate from rapid reproduction (see later).

3.1.2 Brain aging

Brain sections of 9 weeks old *Nothobranchius furzeri* show autofluorescence – when seen under a UV-blue light of a fluorescence microscope – for the presence of the fluorescent age pigment *lipofuscin* (Fig 3.3A), a known autofluorescent aging marker (see *Introduction* chapter).

Through an immunohistochemical assay I showed that a mouse monoclonal antibody which recognizes an epitope of the Amyloid Precursor Protein (APP),[23] which is highly conserved from humans to fishes (80 % in aminoacidic identity between human and zebrafish sequence [24]), detects the presence of APP plaques in 9 weeks old *N. furzeri Optic Tectum* (Fig 3.3B–D).

An antibody specific for the hyper-phosphorilated form of microtubule

[23] MAB 1561, Chemicon.

[24] analysis performed with the NCBI protein-protein BLAST on the URL www.ncbi.nlm.nih.gov/BLAST/

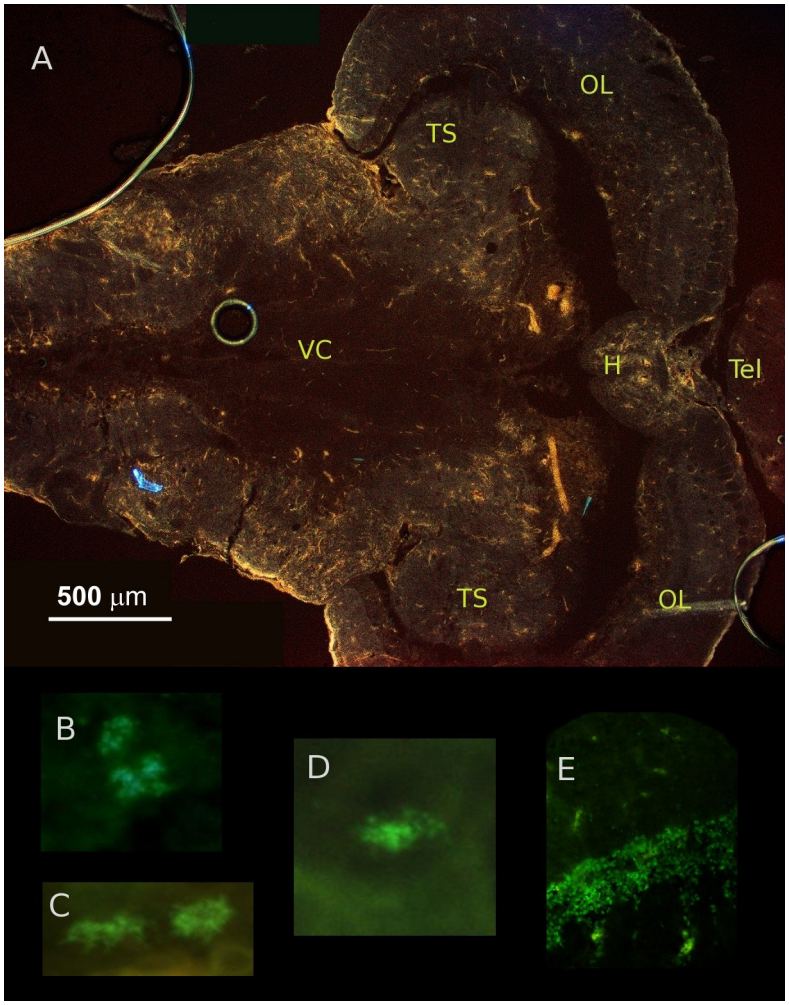

Figure 3.3: Istological markers of brain aging in 9 weeks old GRZ inbred *Nothobranchius furzeri*. (A) Autofluorescence for *lipofuscin* in a sagittal brain section (tail left). (B–D) Amyloid plaques in the *Optic Tectum*. (E) Tau-protein hyper-phosphorylation in the *Optic Tectum*. *OL*: optic lobe of Optic Tectum; *H*: habenula, *Tel*: Telencephalon; *VC*: Valvula Cerebelli; *TS*: torus semicircularis.

associated tau-protein,[25] a classical marker of human *Alzheimer Disease*, produced a strong staining in the *Optic Tectum* of 9 weeks old fishes (Fig

[25] AT8, Innogenetics.

3.3E). Zebrafish tau protein sequence is highly similar (78% identities) to mouse reference sequence. This marker could be used to detect differences in hyper-phosphorylation levels among different age-classes in the fish.

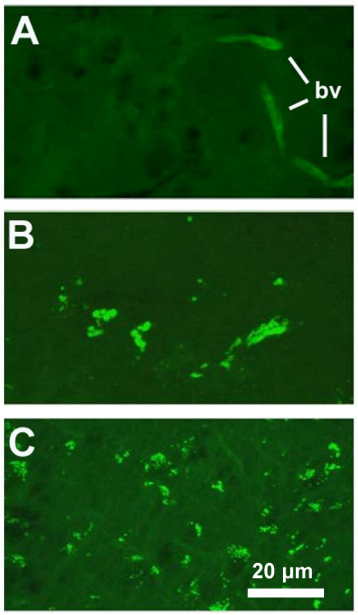

Figure 3.4: Confocal microscope laser detection of (autofluorescent) lipofuscin granules in the brain. (A) *Optic Tectum* of 5 weeks old GRZ inbred *N. furzeri* showing no staining for lipofuscin. (B) *Optic tectum* of 9 weeks old GRZ inbred *N. furzeri* showing lipofuscin granules. (C) Senescent mouse brain (20 months) showing intense staining for lipofuscin. *bv*: blood vessels.

Lipofuscin granules were also detected – with a confocal microscope (488 nm excitation) – in 9 weeks old *N. furzeri* brains (Fig 3.4B). Staining for lipofuscin was absent in young-adult 5 weeks old brains, although visible in blood vessels (Fig 3.4A). Staining for lipofuscin in 9 weeks old *N. furzeri* is sensibly lower than in 20 months old mice brain (Fig 3.4C).

Fluoro-JadeB histochemistry, a specific assay for detecting neurofibrillary degeneration in both apoptotic and necrotic neurons, showed a strong staining in 9 weeks old fishes compared to young-adult 5 weeks old ones (Fig 3.5), suggesting a rapid cytoskeletal degeneration occurring within a time frame of 4 weeks and possibly affecting neural function.

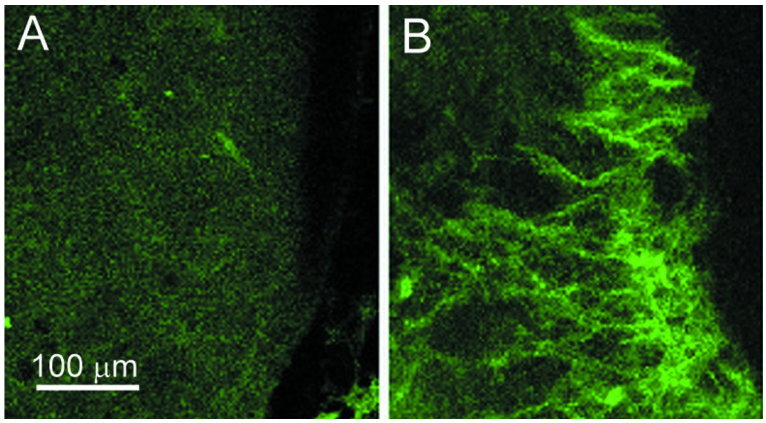

Figure 3.5: Neurofibrillary degeneration in *Optic Tectum* of GRZ inbred *Nothobranchius furzeri* detected with Fluoro-JadeB histochemistry. Horizontal sections of the *Optic Tectum*, after reacting for Fluoro-JadeB were acquired under confocal microscope. (A) Five weeks old young fishes. (B) Nine weeks old *senescent* fishes showing intense Fluoro-JadeB staining.

3.1.3 Peripheral tissues aging

I proceeded to assess whether central nervous system accumulation of aging markers was also paralleled by peripheral tissues aging.

Following the same procedure used by Kishi *et al.* in zebrafish (Kishi et al., 2003), Senescence Associated β Galactosidase activity was detected in fibroblasts of 9 weeks old inbred GRZ *N. furzeri*, whereas it was not de-

tected in 5 weeks old fishes (Fig 3.6). Senescence Associated β Galactosidase is a marker of cellular senescence (see *Introduction* chapter) which was first developed in human cell cultures and skin (Dimri et al., 1995). It is worth mentioning that Kishi et al. detected Senescence-Associated β Galactosidase activity in skin of 31 months old zebrafish, but not in 18 months old fishes. It takes more than 1 year for an adult zebrafish to develop markers of cellular senescence, whereas it takes only 2 months (9 weeks) for *N. furzeri* to show strong evidence of cellular senescence.

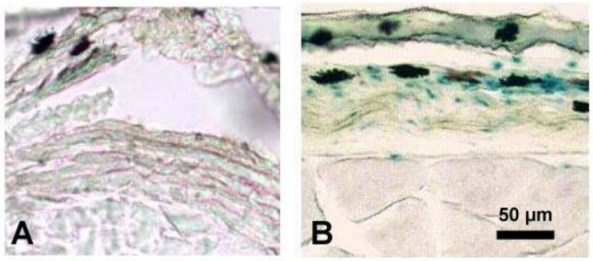

Figure 3.6: Marker of replicative senescence in fibroblasts: Senescence Associated β Galactosidase activity in coronal skin sections of GRZ inbred *Nothobranchius furzeri*. (A) Five weeks old young fish, showing no staining. (B) Nine weeks old *senescent* fish showing staining for Senescence Associated β Galactosidase activity. Black granules are chromatophores, blueish shadows evidence *X-gal* staining for senescent fibroblasts. Scales lie under the epidermis (not visible). Fish epidermis is exclusively composed of alive cells, note the absence of superficial layer of dead, keratinized cells.

To detect if all organs were aging at the same rate, we assayed the age-dependent accumulation of lipofuscin granules (see *Introduction* chapter) in the liver, a good candidate to look at, because it is easy to detect during dissections and is large enough to be successfully handled and processed in surgery and immunohistochemical procedures. Liver is an organ with high

lipid content, high oxidative metabolism and it is therefore subject to oxidative stress. Liver slices taken at 5 and 9 weeks of age and excited with a 488nm laser and then acquired at the confocal microscope showed the same trend already showed for the brain, i.e. also in the liver, 9 weeks old *N. furzeri* are strongly stained for lipofuscin autofluorescence, whereas no signal was detectable in 5 weeks old fishes liver slices (Fig. 3.7), suggesting a generalized trend of aging occurring in the whole organism.

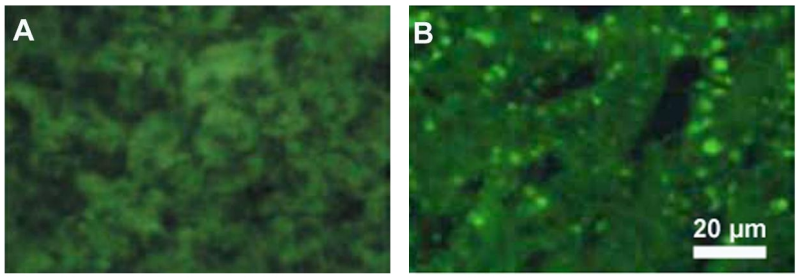

Figure 3.7: Marker of liver senescence in GRZ inbred *N. furzeri*: lipofuscin. (A) Five weeks old fish liver slice, showing no lipofuscin autofluorescence. (B) Nine weeks old fish liver slice, showing random dot-staining for lipofuscin autofluorescence.

3.1.4 Locomotion Decline

In order to look at markers of functional decay, I measured motor decline with two different methods. In the assay first I measured an index of spontaneous locomotion, expressed as the fraction of fishes which were actively moving (swimming) within a time frame of 5 seconds (see *Methods* chapter). Fishes were collectively (10 fishes per tank) video-recorded in their housing tanks with light and temperature similar than their housing conditions. Every week, starting from week 4, fishes were video-recorded with a digital

video-camera by the long sides of the tank for 1 hour. Digital movies were analyzed as explained in *Methods* chapter and and each week a measure of average movement was obtained. I observed a significant age-dependent decline, in particular occurring form week 8 to week 9 (Fig 3.8), when fishes underwent a dramatic decrease in locomotion, changing from a still active behavioral state at week 8 to a semi-immobility at week 9.

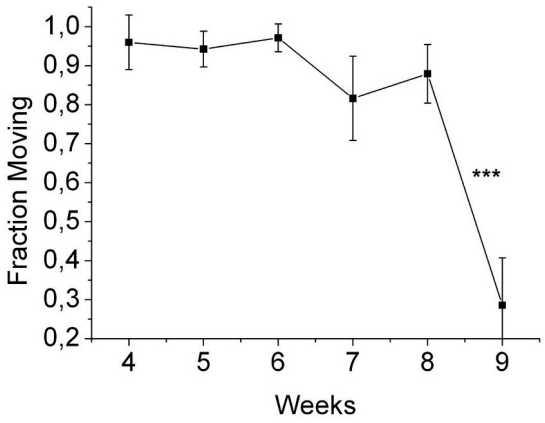

Figure 3.8: Spontaneous locomotion decline in GRZ inbred *Nothobranchius furzeri* in social condition. The measure is computed as the fraction of fishes considered "actively moving" following a criterion explained in the *Methods* chapter. Every measure is computed on 10 fishes. Kruskall-Wallis One-way Anova, ***= $p < 0.001$. Bars are Standard Deviations.

The second method used for measuring locomotion decline consisted in measuring every week the individual fish locomotor activity in a new environment, a 20 liter test-tank where fish explored the new environment for 30 min and were video-recorded from above. Fishes were tracked weekly in groups from 8 to 10. This test is similar to the *Open Field* protocol used in ro-

dents for measuring disposition to explore and anxiety (Crabbe et al., 1999). The acquired movies were analyzed by *Ethovision* Software (Noldus^{TM}, the Netherland) which measured many parameters, the most important being average velocity and percent time spent moving (see *Methods* chapter for details). I found that with this method, similar to what I found with the spontaneuos locomotion, a significant locomotor decline is scored (Fig 3.9), confirming the measure previously obtained and suggesting a decreased capacity to explore new environments and possibly find the way out when chased by natural predators.

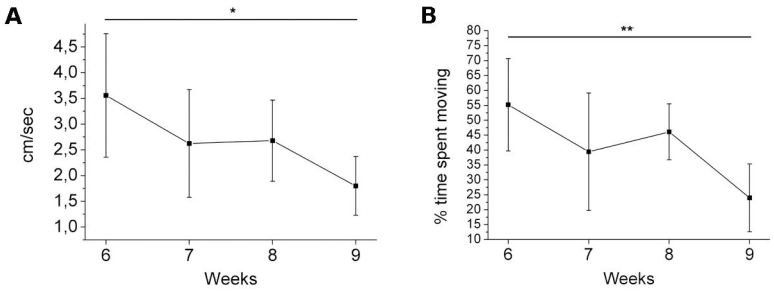

Figure 3.9: Open field test in GRZ inbred *Nothobranchius furzeri*. Average velocity (A) and percentage of time spent moving (B) are scored by the Software *Ethovision* (Noldus^{TM}, the Netherland). In both graphs N= 8-10 for each point. One way Anova, *= $p<0.05$; **= $p<0.01$.

3.1.5 Cognitive Decline

I used a shuttlebox modified by Pradel et al. (Pradel et al., 1999) to measure learning in young and old GRZ inbred *N. furzeri* (see *Methods* chapter for details). The test consisted in training the fish to avoid a mechanical shock by passing through a hole within 15 seconds after the onset of a red light.

Training and testing were simultaneous since in 50 consecutive trials fishes were scored for successful response to the conditioned stimulus (i.e. by passing through the hole within a time-frame of 15 seconds after the light onset) or for failure (i.e. by passing through the hole after being chased by the unconditioned mechanical stimulus occurring 15 seconds after light onset or simply not passing throught the hole at all).

The fishes were scored one by one and each trial duration was 1 minute. Young fishes successfully achieved a significantly higher score than old ones (Fig 3.10). Interestingly, 9 weeks old fishes were still able to learn the test, with a learning peformance significantly higher than the chance level. It needs to be demonstrated that, in this species, cognitive deficit decay, measured in the *Active Avoidance* task, is independent from locomotion decay. In fact, less exploratory fishes could be thought to be less likely to achieve successful trials by chance, moreover extremely inactive fishes cannot achieve anyway a successful trial. As shown in next sections I found a complete dissociation between cognitive decay and locomotion decay in other strains of the same species.

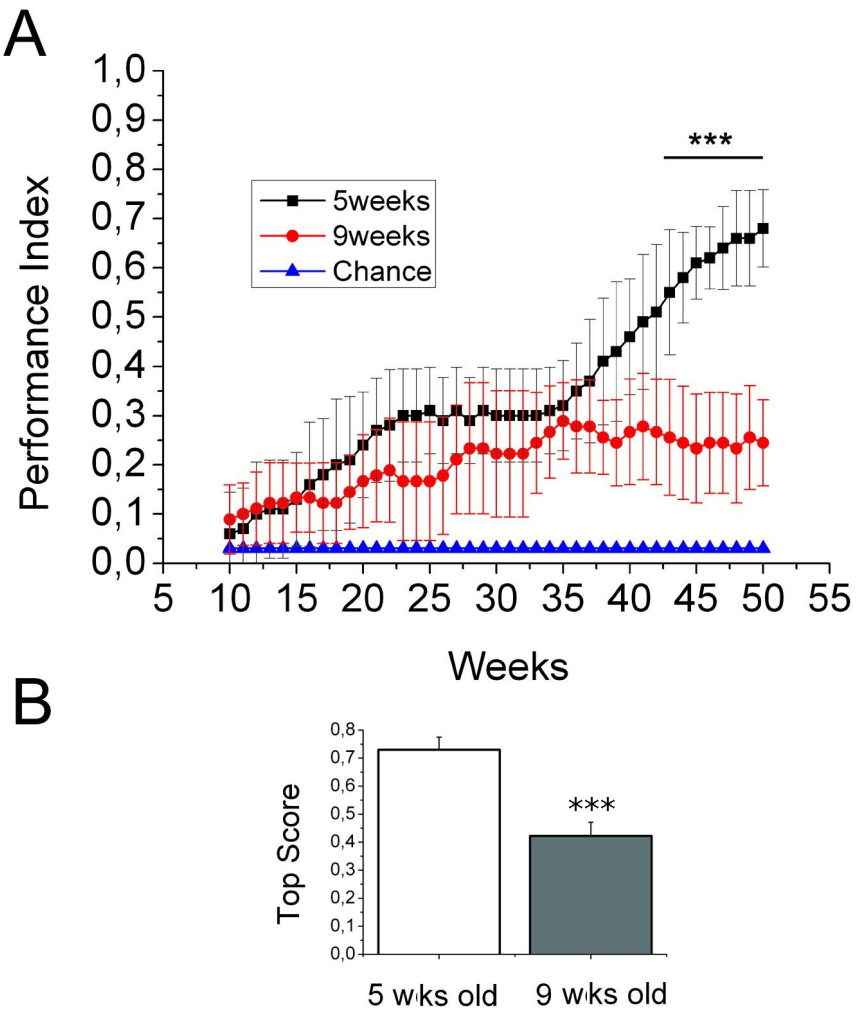

Figure 3.10: Measure of age-dependent cognitive decline: Active Avoidance in GRZ inbred *Nothobranchius furzeri*. (A) Evolution of Performance Index as a function of trial number. Performance index is computed as explained in *Methods* chapter. Chance level is measured as the average score of the first trial (i.e. before the occurrence of the conditioning) of all tested fishes. Two-way Anova. ***= $p < 0.001$. (B) Top Score comparison in the two age groups. Mann-Whitney U test ***= $p < 0.001$. Top Score index is computed as explained in *Methods* section. Five weeks old fishes, N= 10; 9 weeks old fishes, N= 10. Bars are Standard Errors.

3.2 Temperature dependent Survival and Aging

To study the effects of low temperature on fish aging and longevity, we have hatched 246 GRZ inbred *Nothobranchius furzeri* and grown them in standard conditions as explained in *Methods* chapter until week 4. From the beginning of the 5th week of life, we have separated the fish in two esperimental groups: one kept at 25 °C (N= 135) and the other at 22 °C (N= 101).

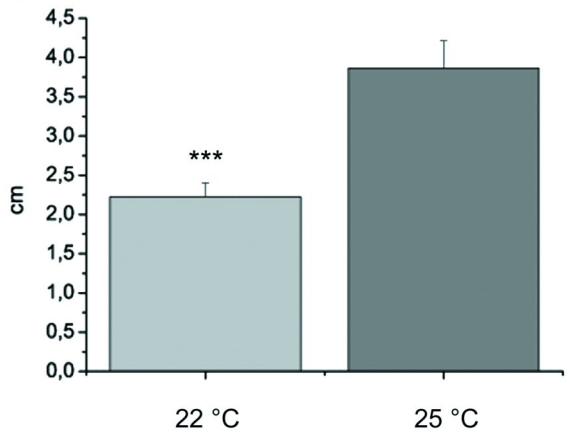

Figure 3.11: Body size of 9 weeks old GRZ inbred *Nothobranchius furzeri* grown at two different water temperatures since 4th week of life. N= 10 for each group. Mann-Whitney U test ***= p< 0.001. Bars are Standard Errors.

Feeding the two groups with a standard protocol (see *Methods* chapter for details), we measured fish standard lengths at 9 weeks of age (Fig 3.11). Fishes kept at 22 °C remained significantly smaller than those kept at 25 °C.

A comparison of survival and death rate for the two experimental groups as well as another group grown at 28 °C (N= 20, only used for survival com-

parison) showed that fishes kept at 22 °C live significantly longer than those kept at 25 °C. In addition, fish kept at 25 °C live longer than those kepts at 28 °C. Furthermore, fish kept at 22 °C have a lower rate of age-dependent increase in mortality (Fig 3.12) than those grown at 25 °C.

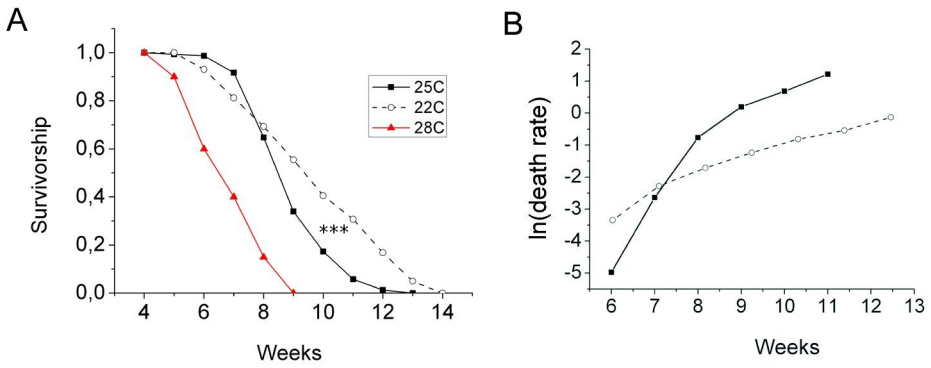

Figure 3.12: Survival and death rate in GRZ inbred *Nothobranchius furzeri* grown at different temperatures. (A) Survival of fishes grown at 22 °C (N= 135), 25 °C (N= 101) and 28 °C (N= 20). Survival at 22 °C and 25 °C significantly differ (Logrank test, ***= p< 0.0001; fishes at 28 °C are not considered for statistical analysis due to the small individual numbers). (B) Log plot of death rate in fishes grown at 22 °C and 25 °C. Note how the slopes differ in the two groups and the intercept of fishes at 22 °C is higher in the first weeks, indicating an early negative effect of low temperature on survival.

The median lifespan [26] of the three groups is 6 weeks for the group at 28 °C, 9 weeks for the group at 25 °C and 10 weeks for the group at 22 °C.

Fishes grown at different water temperature since they are 4 weeks old were also scored for behavioral markers of senescence, like average velocity and percentage time spent moving in an *Open Field*-like test (Fig 3.13).

[26] age when 50% of initial number of fishes are alive

This analysis demonstrated that fishes kept at lower temperature are more active than fish kept at high temperature already at week 5. Moreover, unlike the locomotion decay observed in fishes kept at 25 °C, 9 weeks old fishes grown at 22 °C are more mobile (for both locomotion measures) than 5 weeks old ones.

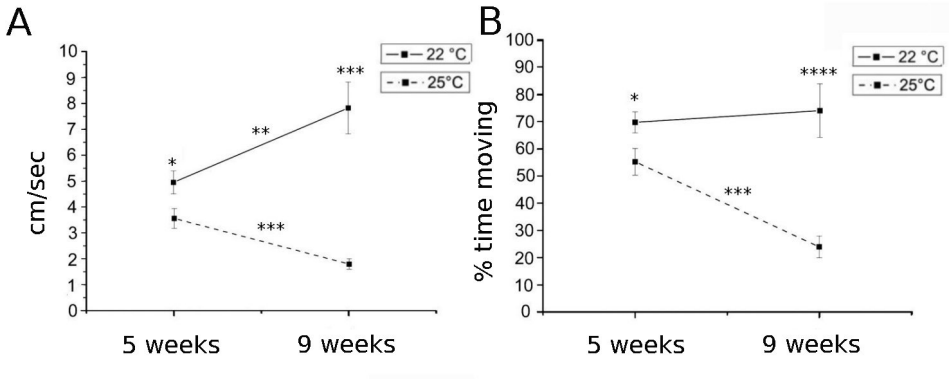

Figure 3.13: Open Field test in GRZ inbred *Nothobranchius furzeri* grown at two different water temperatures: 22 °C (N= 101) and 25 °C (N= 135). (A) Average velocity. (B) Percentage of time spent moving. Motion analysis were performed on digital video files by the software Ethovision. Mann-Whitney U test performed between same age/different group comparisons; Kruskall-Wallis One-Way Anova performed between same group/different age comparison. *= p< 0.05; **= p< 0.01; ***= p< 0.001; ****= p< 0.0001.

Fishes grown at 22 °C were also tested for learning an *Active Avoidance task* with a modified shuttlebox (see *Methods* chapter for details on the technique) when 9 weeks old, an age in which fishes grown at 25 °C had significantly lower scores than 5 weeks old ones from the same group (Fig 3.14). Very interestingly, 9 weeks old fishes which were kept at 22 °C since the completion of the 4th week of life, attained a very performance. It is evident that lower water temperature strongly delays the onset of cognitive

decay. We measured both evolution of performance and top score analysis in the two temperature groups and the result was that fishes kept at lower temperature reached higher scores at 9 weeks of age. Moreover, 9 weeks old fishes kept at 22 °C learned the task faster than 5 weeks old controls, as can be seen in Fig. 3.14A, in fact their performance indexes were higher than those of 5 weeks old controls for the whole plot. In this way, they reached the plateau score faster.

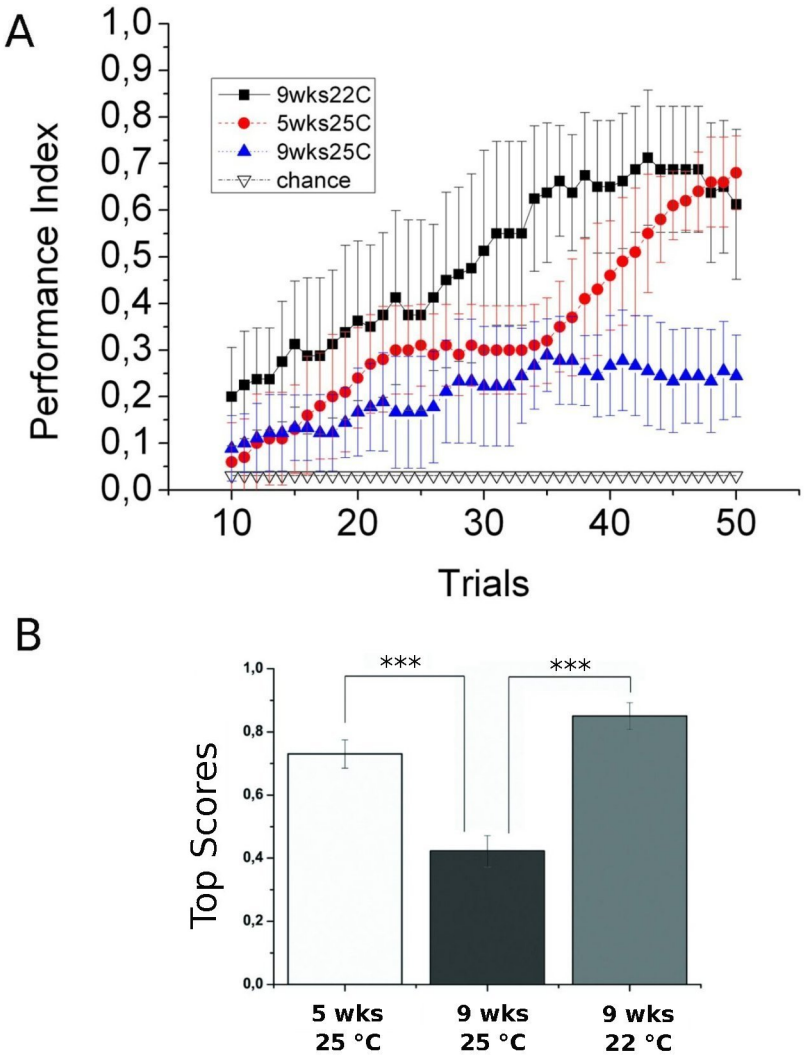

Figure 3.14: *Active Avoidance* in two groups of GRZ inbred *Nothobranchius furzeri* kept at 22 °C (N= 10) and 25 °C (N= 10). (A) Evolution of Performance index score in the two groups. (B) Top Score comparison in the two groups; bars are standard errors. As exemple of highly performing group in (A) and (B) it is reported the score of 25 °C 5 weeks old fishes. Mann-Whytney U test, ***= p< 0.001.

3.3 Effects of *Resveratrol* on lifespan and age-related markers

In order to score the effects of the polyphenol resveratrol in aging and longevity of a short-lived vertebrate, a total of 145 fishes were raised in standard conditions (see *Methods* chapter for details) until the 4th week of life, when they reach sexual maturity (Genade et al., 2005). Throughout the remaining part of their life 110 fishes were fed food supplemented with resveratrol (see *Methods* chapter for details on preparation of resveratrol pellets) at three different concentrations: 24 μg/g food (n= 30); 120 μg/g food (n= 60), and 600 μg/g food (n= 20), while 47 control fishes continued to receive standard food. All experimental fishes received every day 50 mg of food/g of fish weight/day, i.e. were not fed *ad libitum* and were kept at constant temperature and lighting conditions. Resveratrol pellets were not avoided or rejected by fishes, i.e. fishes did not distasted food pellets containing resveratrol but apparently consumed the same amount of control food and resveratrol containing food. This would theoretically exclude the possibility that resveratrol induced an unwanted dietary restriction; anyway, to further exclude this possibility, I compared size in control fed fishes and fishes fed with 120 μg(resveratrol)/g(food) at 9 weeks of age, i.e. after 5 weeks of treatment. This comparison showed no difference (mean control total length =3.86 $\pm$ 0.95 cm; mean total length resveratrol-fed fishes = 3.80 $\pm$ 1.01 cm. Mann-Whytney U test, p >> 0.05; N=10 for each group) in the two groups. It is known, in fact, that dietary restricted animals have smaller size than normal/*ad libitum* fed ones (Anson et al., 2003; Masoro et al., 1982), thus, resveratrol doew not seem to induce dietary restriction.

The analysis of survival showed that fishes fed with the two highest

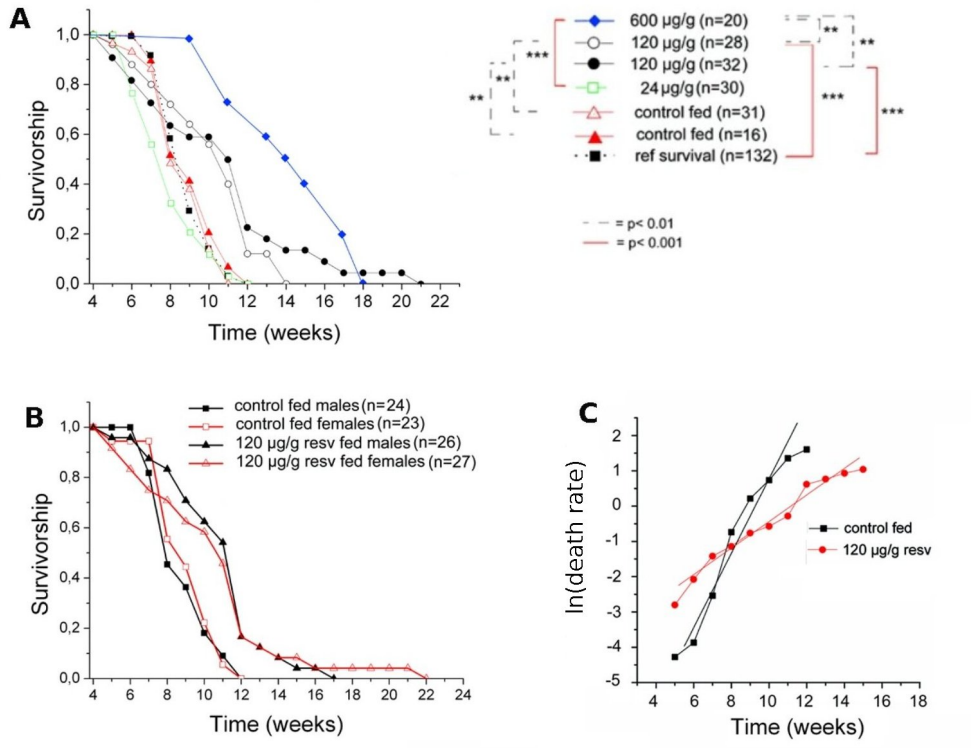

Figure 3.15: Survival and death rate in resveratrol fed GRZ inbred *Nothobranchius furzeri*. (A) Survival curves of five separate experiments and of a reference set for a total of 289 animals. Two trials of control-fed fishes (N = 47); reference survival of untreated fishes (N = 132, a total of 5 replicates); 24 μg/g resveratrol-treated fishes (N = 30); two trials of 120 μg/g resveratrol-treated fishes (N = 60; 4 fishes were sacrificed for histological analysis and censored at age 9 weeks); 600 μg/g resveratrol-treated fishes (N = 20). (B) Comparison between the age-dependent survival of males and females in controls and 120 μg/g resveratrol-treated fishes. Control males (N = 22) and females (N = 25); 120 μg/g resveratrol-treated males (N = 30) and females (N = 27). Three animals could not be sexed upon death. (C) Death trajectories in controls ($y = 1.0452x - 4.4726; R^2 = 0.9492$) and 120 μg/g resveratrol-treated fishes ($y = 0.3761x - 2.3177; R^2 = 0.9739$).

doses of resveratrol (120μg/g and 600μg/g) lived significantly more than control fed fishes, and the ones fed with 600μg/g lived significantly more than those fed with 120μg/g (Fig 3.15).

The two replicates on the 120 μg/g dose and the observed dose dependent effect on *N. furzeri*'s lifespan shown in figure 3.15 (Logrank statistic), put between 24 and 120 μg/g the lower dose of resveratrol able to increase *N. furzeri*'s lifespan. Interestingly, resveratrol appears to have the same effect on males and females lifespan and induces an early mortality which is higher than that observed for control fed fishes, probably inducing an early hormetic effect, then followed by delayed aging. Anyway further data (higher numbers of resveratrol-fed fishes) would be needed to prove this hypothesis, i.e. order to reach a statistical significance.

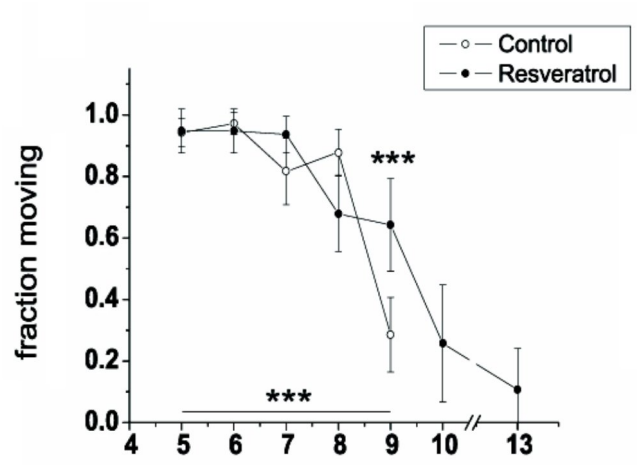

Figure 3.16: Spontaneous locomotion decline in resveratrol-fed (120 μg/g) and control-fed GRZ inbred *Nothobranchius furzeri* in the housing tank (see *Metods* chapter for details). In both groups locomotion decline is higly significant: Kruskall-Wallis, rank-based Anova; ***= p< 0.001. N= 10 for each group. Between the two groups the score at 9 weeks is significantly different: two-way Anova, Tukey post hoc test; ***= p< 0.001.

Resveratrol-fed fishes were tested also for age-associated behavioral traits. For this analysis, we compared 120 μg/g resveratrol-treated fishes with control fed ones. In particular I first looked at the age dependent decrease in spontaneous locomotion in the housing tank and then in the *Open Field*-like exploration.

Fig 3.16 shows that the spontaneous locomotion decline in the housing tank is delayed by one week in resveratrol-fed fishes.

The *Open Field*-like test showed that individual fishes belonging to resveratrol-fed group, instead of undergoing a decrease in motility from week

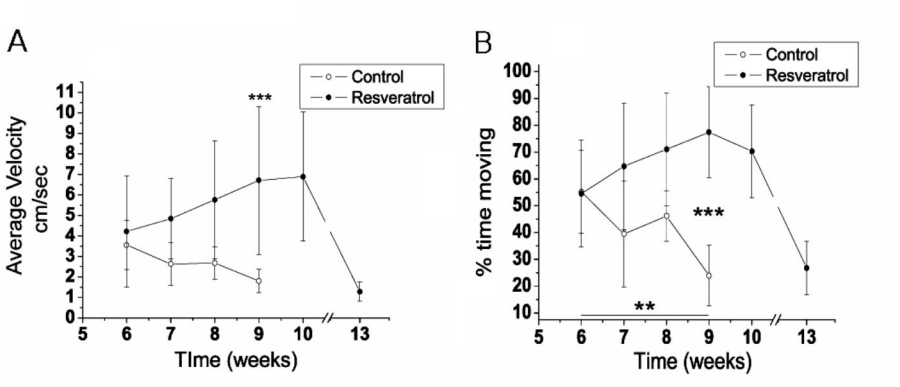

Figure 3.17: *Open Field*-like locomotion test in resveratrol-fed and control-fed GRZ inbred *Nothobranchius furzeri*. (A) Average swimming velocity. (B) Percentage of time spent moving. Bars are standard errors. Analysis were performed by the software Ethovision. Pairwise comparison were performed by Kruskall-Wallis two way Anova, Tukey post-hoc test; **= $p<0.01$; ***= $p<0.001$. N= 10 for each group.

6 to week 9, were more active at week 9 compared to week 6 and their measures at week 9 were significantly higher than those of control fed at week 9 (Fig 3-17). In this case, resveratrol did not produce only a delayed locomo-

tion decay, as in spontaneuos locomotion measure (as in Fig 3.16), but was responsible of an increase in *Open Field*-like exploration during adulthood.

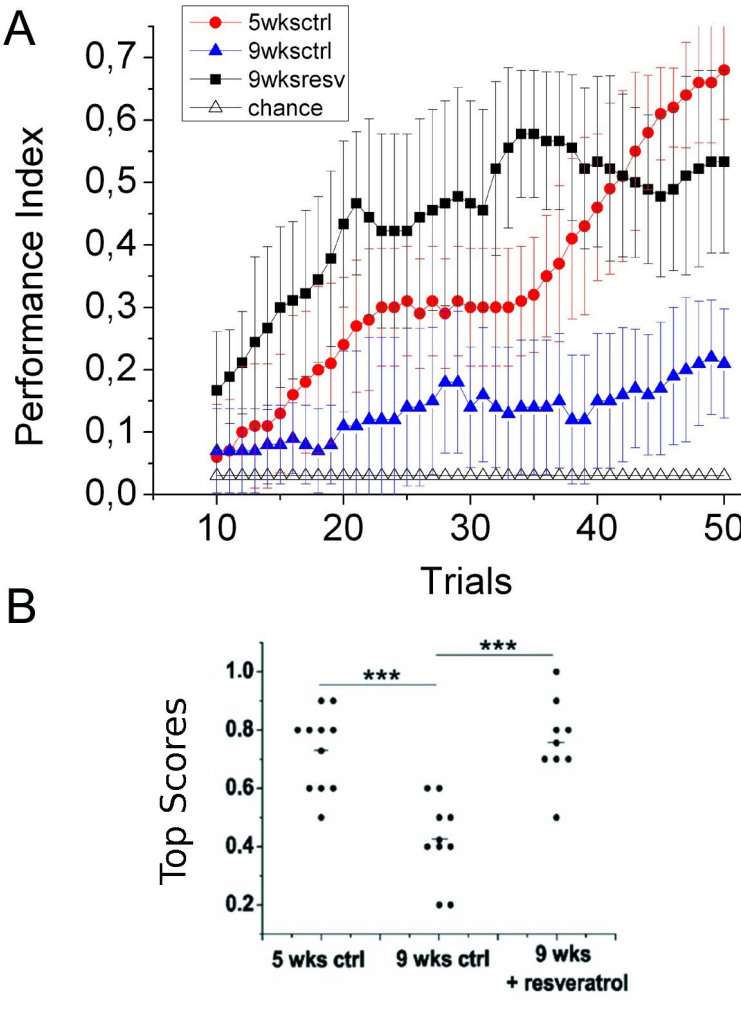

Figure 3.18: *Active Avoidance* task in μg/g(food) resveratrol-fed GRZ inbred *Nothobrancius furzeri*. (A) Evolution of performance (see *Methods* chapter for details) in 9 wo resveratrol-fed; 9 wo control fed and, for comparison, 5 wo control fishes. Bars are standard deviations. (B) Top score comparison among the three groups. Mann-Whitney U test, ***= $p<0.001$. N= 10 for each group.

Fishes fed with 120 μg(resveratrol)/g(food) were also tested for learning an *Active Avoidance task* with a modified shuttlebox (see *Methods* chapter for details on the technique) when 9 weeks old, in order to compare their scores with those of control fishes, which underwent a strong cognitive decline from 5 to 9 weeks of age. I observed a highly significant difference among control-fed fishes and resveratrol-fed fishes at 9 weeks of age, in particular the group of treated fishes did not show any evidence of cognitive impairment in both the measures "Evolution of performance index" (Fig 3.18A) and in comparison of "Top Scores" (Fig 3.18B). Resveratrol-treated 9 weeks old fishes had significantly higher "Performance Index" than 5 weeks old control ones for the first 40 trials of the task (learning phase), then, after reaching plateau, they stablized their scores (Fig 3.18).

We used Fluoro-JadeB as a neuronal marker of neurodegeneration (see *Introduction* and *Methods* chapters for details) to check whether brain structures were rescued in 120 μg/g resveratrol-treated fishes. Fig 3.19 shows fluorescence associated with degenerating neurofibrils and whereas 9 weeks old control *N. furzeri* are strongly stained for Fluoro-JadeB, indicating high levels of neurofibrillary degeneration (Fig 3.19B), 5 weeks old non-treated (Fig 3.19A) and 9 weeks old treated with resveratrol (Fig 3.19C) fishes have significantly lower staining, therefore less degeneration. This last result is the first evidence that *in vivo*, resveratrol prevents neurodegeneration in a vertebrate.

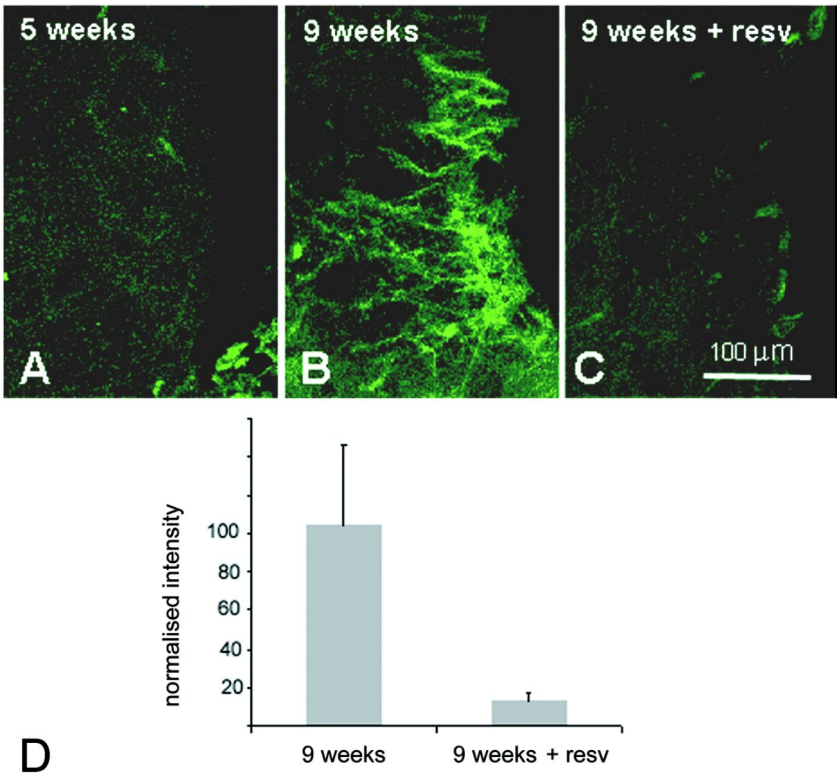

Figure 3.19: Neurofibrillary Degeneration in a Horizontal Section of Stratum Griseum Superficiale of the *Optic Tectum*. The specific reaction product is flurescent green. (A) Five-week-old fish. Note absence of specific signal. (B) Nine-week-old control fish. Note the bright labeling in neuronal processes orthogonal to the tectal surface. (C) Nine-week-old 120 μg/g resveratrol-treated fish. (D) Quantification of labeling intensity. Gray level values are normalized with respect to the average gray value of controls, which is arbitrarily set to 100. Bars are Standard Errors of Means.

3.4 Collection Trip MZM 04

In March-April 2004, together with Dr. Alessandro Cellerino, Stefano Valdesalici, Miles Parisi, Eva Terzibasi, Shayne Fuller and Graeme Ellis, I participated to a collection trip in Mozambique aimed at collecting different wild populations of *Nothobranchius furzeri*. The area chosen for the collection was the lower Limpopo drainage system, where several *Nothobranchius furzeri* wild populations were found as stated by a recent report by Trevor Wood (1999). This area is characterized by a meteorological gradient of precipitations which are abundant on the Indian Ocean Coastline

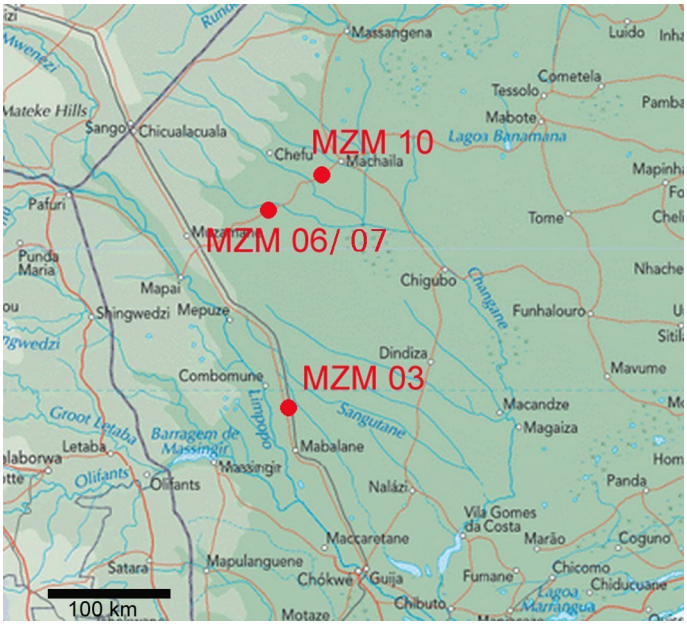

Figure 3.20: Map of the area sampled during the collection trip MZM04. In red are indicated the localities which provided the populations which were successfully reproduced in captivity.

(the city of Xai-Xai has 1050 mm rain per year), but very scarce near the

border between Mozambique and Zimbabwe (the city of Beitbridge has 350 mm rain per year).[27] We were interested in collecting populations from different climatic regions, from the more humid region close to Chockwe, where a red-tailed populations was collected by Mr Trevor Wood in 1999 (see *Introduction* chapter), to the region adjacent to the border with Zimbabwe, very close to the Gonarezhou National Park, where the GRZ strain was colleted in 1968 (Jubb, 1971). Our expectation was that populations deriving from more humid regions have longer captive lifespan than those coming from the driest localities.

The collection trip was planned and organized during Summer and Autumn 2003, before the occurrence of the forecasted wet season, usually happening in a time frame which goes from December to April. One month after the weather reports (www.weatherunderground.com) referred rainfalls in the district of Chicualacuala, on the south border between Mozambique and Zimbabwe, we epected to find spawning *Nothobranchius furzeri* all over the province of Gaza and in the mozambiquean area adjacent to the border with Zimbabwe. A simple equipment, consisting of two 4x4 wheel drive, camping equipment, fishing nets, boots and fish bags, was enough to achieve a successful collection in populated natural pools. Wild-caught fishes were moved to fish bags (2-3 per bag) with 3 cm of water and a larger volume of air (about 10 times as much as water). Water was changed daily and dead fishes were removed from the bags in order to avoid water pollution. Fish bags were kept in dark and cool places, possibly far from heat sources. After being caught and until the eventual housing in the lab fishrooms, fishes were not fed at all (from 4 days to 8 days). The GPS coordinates (SE) of the localities chosen for sample collections are reported in tab 3.1.

[27] informations got from the URL: www.worldweather.com

Locality Code	GPS Coordinates	N. of Fouders	Species
MZM04-01	24 12.90	–	*N. furzeri*
	32 50.04		
MZM04-02	23 59.06	3f, 1m	*N. furzeri*
	32 36.10		*N. rachovii*
MZM04-03	23 88.52	8f, 5m	*N. furzeri*
	32 36.01		*N. orthonotus*
			N. rachovi
MZM04-04	21 56.17		
	31 49.00		
MZM04-05	22 33.02		
	32 27.58		
MZM04-06	22 30.49	1f, 1m	*N. furzeri*
	32 33.03		
MZM04-07	22 27.03	–	*N. furzeri*
	32 38.83		*N. rachovii*
MZM04-08	22 21.80	–	*N. furzeri*
	32 41.86		*N. orthonotus*
MZM04-09	22 21.75		
	32 43.55		
MZM04-10	22 21.81	1f, 1m	*N. furzeri*
	32 44.39	2f, 1m	*N. orthonotus*
		5f, 2m	
MZM04-11	23 11.61	–	*N. furzeri*
	32 29.21		

Table 3.1: Locality-Codes and GPS (SE) coordinates, Number of founders individuals from each locality and species found in the sampled localities during *2004 MZM04 Nothobranchius furzeri Collection Trip*. Underlined are localities from which the sampled populations were taken back in Italy and successfully reproduced in the Neuroscience laboratory of Scuola Normale Superiore in Pisa. The numbers of founders individuals are referred exclusively to *N. furzeri*. From locality MZM10, 3 groups of founders were kept separeted and were called population *pair*, *trio*, and *group*, depending on the number of the founders.

3.5 Aging in wild derived *Nothobranchius furzeri* populations

Wild captured populations were succesfully reproduced in the same captive conditions of GRZ inbred line (see *Methods* chapter for details on standard housing conditions), and the captive longevity of F2 generations was measured for survival (Fig 3.21).

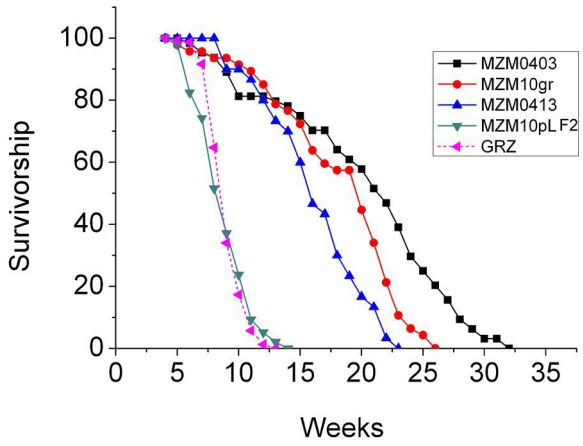

Figure 3.21: Survorship in 4 wild-derived strains of *Nothobranchius furzeri* and in the GRZ inbred line. All but one wild captured populations have significantly higher longevity than GRZ inbred line (Logrank test $p<0.001$). MZM10pL strain survivorphip does not differ from the GRZ inbred line. MZM0403, N=58; MZM10gr, N= 47 (comprising population *pair* "early" (see text), *trio* and *group*); MZM0413, N= 30; MZM10pL, N= 91.

The reported survival curves show that, among the wild populations that we have reproduced and studied in Pisa (including the population *MZM0413* collected by Dr. Brian Watters in a location close to MZM0403), only one

is characterized by a survivorship comparable to that of the GRZ inbred strain: population *MZM10pL*. Interestingly, this population comes from the location that, among those that we sampled, is situated much closer to the

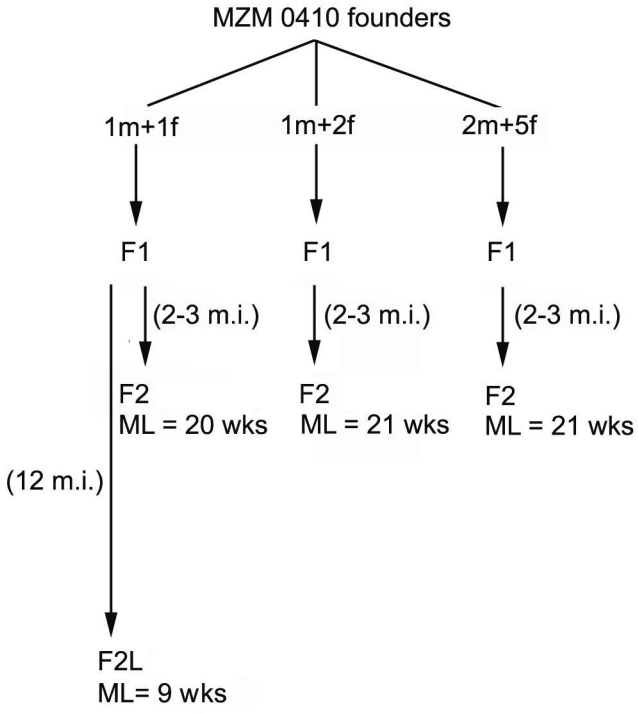

Figure 3.22: Derivation of 4 captive strains from locality MZM10. Note that we derived a short-living F2 strain (9 weeks of median lifespan) from long-incubation F2 eggs layed by the F1 of the *pair* founders (see Table 3.1). The same F1 generation produced short-incubation eggs which generated a long-living strain (20 weeks of median lifespan). m.i. = months of incubation. ML = median lifespan.

border with the GRZ National Park in Zimbabwe, where *Nothobranchius*

furzeri was discovered in 1968 by Furzer and Warne (Jubb, 1971). Population *MZM10pL* is derived, as indicated in Fig 3.22, from a pair of founders.

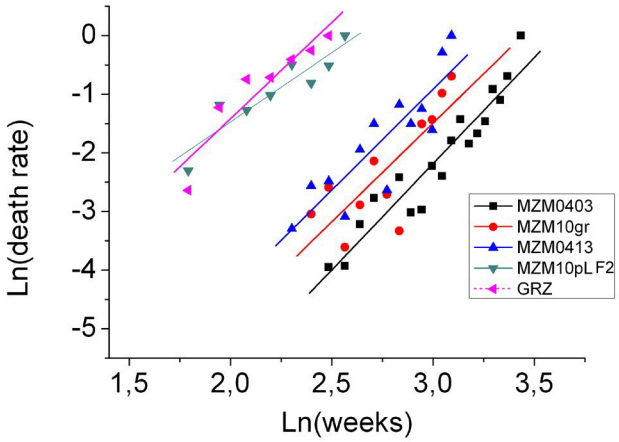

Figure 3.23: Death rate log-plot of wild-derived and GRZ inbred *Nothobranchius furzeri*. Linear interpolations are computed excluding the first 10th percentile from the survival distribution. In order to linearize the late-life death-rate phase also the log of X-axis was computed as indicated by Reznick *et al.* (Reznick et al., 2004). Note the same slope and the different intercepts among the various interpolations.

In the F2 generation two types of eggs were produced: (A) fast-developing eggs gave rise to fishes with a median lifespan of 20 weeks; (B) eggs with prolonged diapause gave rise to fishes with an aging phenotype similar to GRZ inbred (Fig. 3.22). Two more lines were derived from the MZM10 location (Table 3.1), both gaving rise to fishes with a median lifespan of 20-21 weeks. The survival data of these populations are collapsed as MZM10gr (Fig. 3.21, 3.23). In order to compare the aging rates of wild derived and the GRZ inbred strain, I log-plotted the death rates derived from the

survival curves showed in Fig 3.21.[28] Fig 3.23 shows that different populations share the same slope in death-rate curves but different intercepts, i.e. the age-independent increase in death rate is comparable among populations, but different populations differ for the timing of the onset of the death risk increase.

Wild-derived populations were also scored for functional markers of senescence, i.e. learning and locomotion decline (Fig. 3-23).

Behavioral measures of age-dependent functional decay show that all but one tested populations undergo an age-dependent cognitive decay between 5 and 9 weeks. In fact, the population *MZM0403* does not achieve significantly lower scores at 9 compared to 5 weeks. It is worth mentioning that population *MZM0403* is also the one with higher median and maximum lifespan (23 and 28 weeks respectively). Another peculiar characteristic of population *MZM0403* was that it was the only one, among the studied populations, where the large majority of adult males displayed a red tail fin.

Interestingly, *Active Avoidance* scores are not correlated to the locomotion measures of behavioral decay. The only population showing a concomitant cognitive and locomotion decay is the *GRZ inbred*. F1 MZM10pL and F2MZM10gr show cognitive decay without locomotion deficit.

[28] death rate dr computation from survival (S): $dr_{t+1} = \frac{S_t - S_{t+1}}{S_t}$ for all t (i.e. weeks).

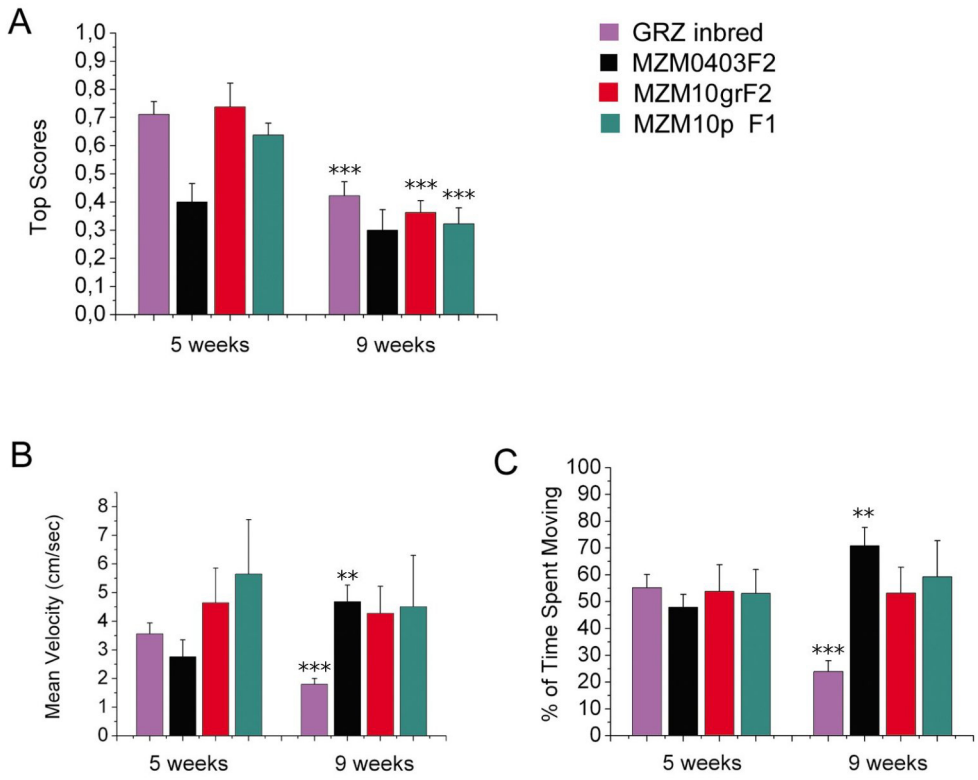

Figure 3.24: Age-dependent decline in learning and locomotion performance (see *Methods* chapter for details) in wild and GRZ inbred *Nothobranchius furzeri*. (A) Top scores comparison in the *Active Avoidance*-test. (B-C) Scores in the *Average Velocity* and *Percentual Time Spent Moving* measure performed by the software Ethovision. Note that the MZM10pL population is a F1 population. Kruskall-Wallis one-way Anova, **= $p < 0.01$; ***= $p < 0.001$. Significance marks refer to pairwise comparison between different age classe of the same population. Bars are standard errors. For each group N= 10.

3.6 Hybrid-line generation.

We crossed *MZM03* females with GRZ inbred males and *MZM03* males with GRZ inbred females, in order to test inheritance of the *longevity* trait (Fig 3.25). We did these two-way crosses in order to detect possible sex-linked inheritance of the *longevity* trait.

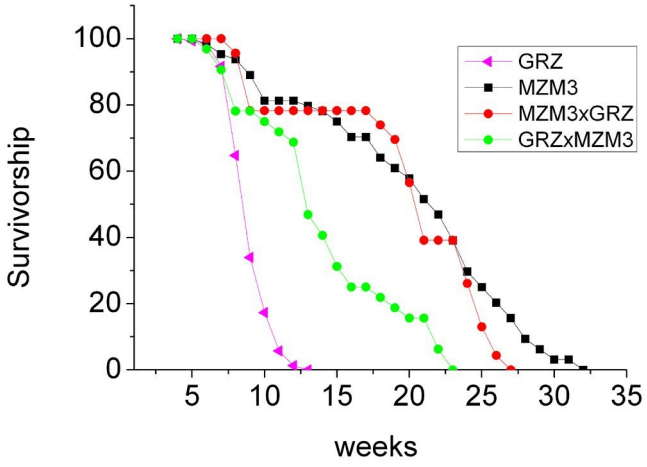

Figure 3.25: Survivorship in two hybrid F1 generations derived from the cross between MZM03 wild-derived line with the GRZ inbred line. In the legend the female is put first, i.e. MZM03xGRZ is the cross of female MZM03 with male GRZ inbred. MZM03xGRZ N= 28; GRZxMZM03 N= 32. GRZ and MZM03 survivorships are the same as in Fig. 3.21.

Both F1 hybrid lines live significantly more than GRZ parental line (Logrank test, $p < 0.001$) and GRZ female x MZM03 male lived significantly less than MZM03 female x GRZ male (Logrank test, $p < 0.01$), but larger numbers of individuals are needed to reach statistical significance (Fig 3.25).

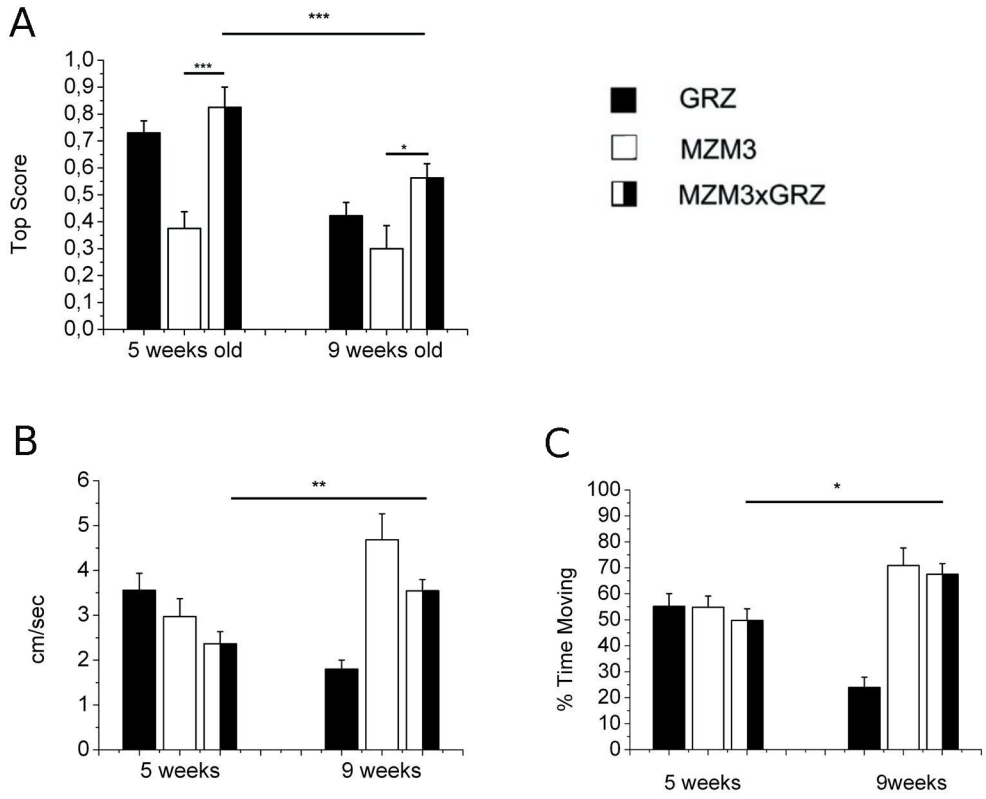

Figure 3.26: Age-related behavioral traits in MZM03 female x GRZ male F1 hybrid line. (A) Top Scores in the *Active Avoidance task* (see *Methods* chapter for details). (B-C) Average velocity and percentual time spent moving in the *Open field*-like test. One-way Anova, *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$. For each group N= 10.

We scored the functional decay of the MZM03 female x GRZ male F1 cross as described in previous sections (see *Methods* chapter). We found that hybrids underwent a cognitive decay from the 5th to the 9th week of (One-way Anova, $p< 0.001$) age but, differently for the maternal line, reached high scores at 5 weeks (Fig 3.26A). Moreover, they did not show a locomotor decline from 5 to 9 weeks of age (Fig 3.26B-C), like the parental

line but, like maternal line, underwent a significant increase both in mean velocity and percentual time spent moving (Fig 3.26B-C).

3.7 Anecdotal Considerations regarding aging *N. furzeri*

Beyond the age-associated markers and traits described in the previous sections of *Results* chapter, it is also worth mentioning some age-related features that I qualitatively observed but did not yet quantify.

Old fishes look paler than young ones, expecially males, which often loose the typical bright sexual coloration of the tail and of the other fins. Old males and females loose weight, and expecially females loose their typical ventral swelling which indicates the presence of eggs. Concerning the GRZ inbred strain, at the age of 9 weeks, females are still able to lay eggs and males to fertilize them, although this is their median lifespan, i.e. the age at which half of initial fish are still alive. Interestingly, reseratrol-fed fishes still laid fertilized eggs at 12 weeks of age. Concerning the wild populations, we observed that individuals older than 11 weeks rarely lay eggs and look very weak and slow, usually they remain still on the bottom of the tank. This condition can last for several weeks.

Very interestingly, adult and old *N. furzeri* and *N. orthonotus*, often develop big bulging bodies on the foremost extremity of the *premaxilla* and mandible. These abnormal structures usually get larger with time, eventually affecting the inner skeletal structure of the mouth. We believe this structures to be tumoral-like bodies, but a proper histopathological analysis deserves to be done. Tumorigenesis has been in any case already reported for fish of the genus *Nothobranchius*, e.g. in *N. guentheri* (Cooper et al., 1983; Markofsky and Milstoc, 1979).

Chapter 4

Discussion

In this thesis I present data which evidence the potential offered by the oviparous Cyprinodontiform Aplocheilidae *Nothobranchius furzeri* as a vertebrate model for the study of aging and longevity. Some of the data I present in this thesis are already published by Dr. Cellerino's group in Pisa (Valdesalici and Cellerino, 2003; Genade et al., 2005; Valenzano et al., 2006).

In particular, I characterized longevity and age-related traits in the GRZ inbred strain, a population derived from the Gonarhezou National Park in southern-east Zimbabwe and reproduced in captivity for about 30 years by hobbyists before reaching a scientific laboratory in 2002. Moreover I also present data regarding wild populations that were captured in Mozambique in March-April 2004, during a collection trip organized by Dr. Alessandro Cellerino's team from Pisa. Datas are also presented regarding survival of one wild-derived population captured in Spring 2004 during another expedition organized in Mozambique by Dr. Brian Watters.

4.1 Aging in GRZ inbred strain of *Nothobranchius furzeri* strain

Model organisms used in aging-research respond to different needs, short living organisms, for instance, are very important for providing insights into the molecular pathways and general mechanisms responsible for changes in aging rate and longevity, since they allow to run many experimental trials in a very short time-frame. The yeast *Saccharomyces cerevisiae* and invertebrate animals like the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster* have been extensively used for finding treatments able to extend/reduce lifespan (Kenyon, 2005) and were fundamental to uncover the importance of the Insulin/IGF1-like pathway as a general pathway responsible for regulation of lifespan in organisms as different as worms and mice (Kenyon, 2005; Tatar et al., 2003). Unfortunately, short living animal models are phylogenetically very distant from vertebrates like *Homo sapiens*, and sometimes they are not very useful as models for human-like age associated traits, like tumors.[29] In addition, model organism can have aging mechanisms which are not shared by vertebrates, like the accumulation of Extra-chromosome rDNA Circles (ERCs) (Sinclair and Guarente, 1997) which cause replicative senescence in the yeast *S. cerevisisae*.

Conversely, vertebrate models like mice, rats and monkeys, are phylogenetically closer to humans, when compared to the mentioned short-living models, but have the inconvenient of being long-lived, e.g. mice live at least 2.5 years (Holzenberger et al., 2003) and therefore require long-lasting experimental trials for assessing the role of any given treatment on lifespan

[29] *Caenorhabditis elegans* and *Drosophila melanogaster* adults, excluding gonads, have all postimitotic tissues.

duration.

In this thesis I describe age-dependent survival and age-associated markers in a short-lived vertebrate: a fish whose longevity is comparable to that of a wild derived *Drosophila melanogaster* population (Fig 3.1A). GRZ inbred *Nothobranchius furzeri* have a peak in absolute mortality at age 9 weeks (Fig 3.1B), which is also the age corresponding to their median survival (Valdesalici and Cellerino, 2003). The accelerated growth observed in this species has been linked to the very dry conditions of its natural habitat (Genade et al., 2005), where the water is usually available for few months each year. For the most part of the year the pools in which *N. furzeri* live are completely dry and the embryos can survive to this harsh conditions by entering and remaining in diapause. When the rainfalls eventually occur[30] the fries hatch very quickly and reach soon sexual maturity. In this way they achieve reproduction before their pools dry up again. This ecological constrain would provide a very likely selective advantage for individuals with very rapid growth and sexual maturation. In support of this hypothesis there is the evidence that within the same genus, species coming from more humid regions, like *Nothobranchius orthonotus* var. *kuntae*, which live close to Beira, in coastal Mozambique, with rain seasons lasting for at least 8-9 months instead of 3 months like in the region of Gonarhezou in southern Zimbabwe, has a slower growth rate (Fig 3.2B). Long lasting water availability, therefore few months (if any) of water scarcity, would reduce selective pressure for rapid development (Fig 3.2). The association between very short lifespan and fast growth in *N. furzeri*, and longer lifespan and slower growth rate in *N. orthonotus* (Fig 3.2A-B), would be in agreement

[30] or when the water is poured in the hatching tanks by the experimenter.

with the following prediction stated by George Williams in his "Antagonistic Pleiotropy theory of aging" (Williams, 1957) (see *Introduction*):

- *Rapid individual development should be correlated with rapid senescence*

It should be worth studying in more details the relationships among growth rates and longevity on a larger number of species and, also, in different populations of the same species which strongly differ for life expectancy.

In this thesis I show that, in a time frame of only 4 weeks, from week 5 to week 9, GRZ inbred *N. furzeri* undergo a process of rapid and global senescence, i.e. not a failure concerning only one system, but a global physiological decay. I consider 9 weeks old *N. furzeri* as senescent, since they reach their median lifespan and present a whole set of histological markers of aging. Lipofuscin autofluorescence, a classical marker of peroxidation associated to aging (Brunk et al., 1992), is present at 9 but not at 5 weeks of age in the brain (Fig 3.3A; Fig 3.4) and in the liver (Fig 3.7). Moreover, some of the typical markers of vertebrates aging brains, like amyloid plaques and tau-protein tangles, aslo important markers of human neurodegenerative diseases like *Alzheimer Disease*, are present in *N. furzeri* at 9 weeks of age (Fig 3.3B-E). Accumulation of amyloid plaques has already been observed in 3-4 years old brains of the spawning pacific salmon (Maldonado et al., 2000), a semelparous fish [31] which dies soon after reproduction, and for which a genetically programmed aging clock has been postulated (Dickhoff, 1989). Also tau proteins have been reported to accumulate in senescent goldfish and zebrafish (Tomasiewicz and Wood, 1999) but none of these models present

[31] with only one reproductive season.

these same markers onset as soon as at 9 weeks of age. *N. furzeri* aging is therefore striking for its rapid occurrence.

Very interestingly, up to date, *N. furzeri* is the only short living model organism to show cellular senescence as early as 9 weeks of age (Fig 3.6). In fact, model organisms with lifespan similar to that of *N. furzeri*, like nematodes and flies, have adults with mostly post-mitotic tissues (i.e. excluding gonads, they do not have renewable tissues) and do not need to balance cell cycle away from out-of-control proliferation (therefore high risk for hyperproliferative diseases) on one side, and away from cell cycle arrest on the other side (Campisi, 2005a; Campisi, 2005b). Cellular senescence has been proposed as an efficient mechanism for lowering the risk of neoplastic transformation in renewing tissues, like fibroblasts. A possible side-effect (pleiotropic effect) of cellular senescence is the lowered capacity of the tissue to undergo renewal, repair and, also, regeneration (Campisi, 2005b). *N. furzeri* show an early onset of replicative senescence (Fig 3.6) (Genade et al., 2005) in fibroblasts (Dimri et al., 1995) which could be considered as a symptom of reduced capacity to respond properly to cumulative environmental stress. This observation underlines the great potentiality offered by *N. furzeri* to be developed as an animal model for cancer research.

Age-related behavioral impairments are observed in models as different as worms (Johnson, 1987), flies (Tamura et al., 2003; Horiuchi and Saitoe, 2005), rodents (Liu et al., 2002) and humans. Behavioral functions are very integrated biological outputs, good candidates to look at when interested in measuring a process of generalized and gradual functional change, like that occurring during aging. I devised three behavioral assays for scoring age-dependent decay in *N. furzeri*. From the 5th to the 9th week of age I

found a significant decrease in behavioral mesures of locomotor activity (Fig 3.8; 3.9), both in social condition and in the *Open Field*-like assay. Moreover I observed a reduced capacity to learn an *Active Avoidance* task (see *Methods* chapter for a detailed description of the test) from the 5th to the 9th week of age (Fig 3.10). These behavioral age-associated traits provide the indication that *N. furzeri* is subjected to a global age-dependent progressive dysfunction, and that histological markers of aging are also related to functional impairments.

4.2 Temperature Dependent Survival and Aging

Temperature variations are known to affect organisms biological processes given the very basic fact that life, compared to other physical events, is possible within a limited range of temperatures. Life mostly depends on the maintainance of the liquid state of cellular and extracellular fluids, which allow the occurrence of biological reactions. The kinetic of enzymatic reactions is dependent on temperature, moreover many molecules have a temperature-dependent conformation which affects their function and ultimately influences the possibility for life processes to occur. Cellular membranes, for instance, are extrimely sensible to temperature variations and need to maintain their fluidity as much as possible, in order to keep their functionality (Hubert and Else, 2005). Since environmental temperature variations are very likely to occur during an organism's lifespan, organisms evolved several mechanisms to face these changes and keep homeostasis. For instance, producing less or more heat at cellular level through leaking protons in the mitochondrial electron transport chain, is responsible by one side for the heat production and, at the same time, for a reduced production of Reactive Oxygen Species (ROS), which are among the principal cellular damaging agents which contribute to aging process (Tuma, 2001; Wallace, 2005). It is therefore to be expected that organisms have adaptive genetic programs evolved in response to temperature fluctuations and to modify body metabolism to external temperature. Differently from Homeotherms, which keep a constant inner temperature by cellular heat production, poikilothermic organisms, i.e. organisms whose inner temperature fluctuates depending on external temperature and physiological activity, like fishes, usually adopt behavioral thermoregulation, i.e. choose to spend more time

in environments with a better thermic climate for given physiological requirements, or adopt behaviors which induce variations in inner temperature (Randall et al., 1999). These metabolic or behavioral changes are linked to a temperature-dependent differential gene expression (Podrabsky and Somero, 2004; Malek et al., 2004; Gracey et al., 2004). Interestingly, temperature variations in poikilotherms are linked to modulation of longevity, given that transitions from higher to lower temperatures induce lifespan increase in many models (Liu and Walford, 1975; Van Voorhies and Ward, 1999; Mair et al., 2005). The mechanisms underlying temperature dependent lifespan modulation are widely explored and it was proposed that lowering ambient temperature affects lifespan through modifying the "rate of aging", i.e. the time dependent accumulation of cellular and tissue damage (Walford et al., 1969; Mair et al., 2005).

We observed pleiotropic effects of environmental temperature variations of 3 °C from standard conditions (25 °C) in the GRZ inbred *N. furzeri*. At low temperatures growth was significantly reduced (Fig 3.11), confirming data which link genes which control cell-growth and proliferation to environmental temperature variations (Podrabsky and Somero, 2004). Life expectancy was inversely modulated by temperature variations (Fig 3.12), confirming similar previous data reported for other poikilotherms (Liu and Walford, 1975; Van Voorhies and Ward, 1999; Mair et al., 2005). Noteworthy, the lower temperature induced an early higher mortality compared to standard conditions, whereas the slope of death rate curve is less steep than in controls, indicating a possible effect on longevity induced by lower temperature through lowering the age-dependent death-risk increment (Mair et al., 2005). This process could be related, for instance, to a lower rate

of production of Reactive Oxygen Species (ROS), as indicated by a recent evidence that, in Zebrafish, temperature reduction induces up-regulation of gene functional groups related to *Oxygen and Reactive Oxygen Species Metabolism* and *Response to Oxydative Species* (Malek et al., 2004; Gracey et al., 2004). Very interestingly, age-related behavioral traits like locomotor and cognitive decay were strongly affected by temperature. In particular, low temperature (22 °C) not only induced a rescue from the locomotor decay occurring from week 5 to week 9 in control fishes (Fig 3.13), but was also responsible for a significant increase in average velocity in a *OpenFeld*-like test. The effects induced by the lower temperature were even more striking when we looked at age-dependent cognitive decay, which was completely abolished in 9 weeks old *N. furzeri* kept at lower temperature (Fig 3.14).

We do not know yet the mechanisms underlying the complete rescue of behavioral decay occurring in fishes grown at lower temperature, since our data on *N. furzeri* are the first to show this correlation. In order to determine whether lower oxidative stress is correlated to the delayed aging phenotype, it would be fundamental to measure, in fishes kept at 22 °C, the levels of oxydative damage in tissues with high metabolic activity, like the liver and the muscles, as well as the brain. At present we do not know whether low temperature acts only through lowering oxidative stress, but this field of studies could greatly benefit from a model like *N. furzeri* for assessing the link between environmental temperature variations and lifespan increase.

Facing environmental temperature variations is a key problem for organisms evolution, including humans. Human mitochondrial DNA haplotypes diversification has been associated to different climatic adaptations happened during human diffusion in the globe (Wallace, 2005). Modern

quasi-epidemic raise in age-related metabolic and degenerative diseases has been related to the rapid changes in the interplay between mitochondrial haplotypes (energetics) and diet (environment), the second being changed to higher calorie contents of diets occurred in western societies since the XX century (Wallace, 2005). Mitochondrial energetics, which depends on calorie content and is also responsible for heat production (Wallace, 1999), seems, at present, the key issue for understanding how low temperatures and low-food diet can affect organisms' longevity and, as our experiments have demonstrated, behavioral decay. It would be therefore interesting to explore how, at different ambient temperatures, variating caloric food intake, would change longevity and age-related behavioral decay.

4.3 Effects of Resveratrol on lifespan and age-related markers

Small molecules were identified which increase lifespan of model organisms when administered with food. Some of these compounds, like ethoxyquin (Comfort et al., 1971), have toxic effects, and are able only to increase lifespan of short-lived mice strains. Other, like deprenyl (Knoll et al., 1989; Jordens et al., 1999), are considered *control substances* for their psychoactive effects. Further compouds, like 4-phenylbutyrate (PBA), revealed to increase flies lifespan in a dose-dependent manner, but the active dose differed between two different strains of flies (Kang et al., 2002), and higher doses were toxic. PBA induces a strong transcriptional activation and inhibits hystone deacetylases (Kang et al., 2002). The antioxidants Lipoic Acid (Bauer et al., 2004), butylated hydroxytoluene (Sharma and Wadhwa, 1983), N-acetylcysteine (Brack et al., 1997), sodium solenite (Kaur et al., 1989), sodium hypophosphite (Wadhwa et al., 1986), were all found to increase fly lifespan. In nematodes, it was reported that catalase mimetics can induce life extension (Melov et al., 2000), but further experiments did not confirm this datum both in worms (Keaney et al., 2004) and flies (Bayrne and Sohal, 2002). Ginko biloba extract EGb 761 (Wu et al., 2002) and vitamin E (Harrington and Harley, 1988) were also reported to increase worms' lifespan. These results on the effect of antioxidant on lifespan increase are in agreement with the mitochondrial theory of aging, which underlines the importance of the mitochondrial-dependent ROS generation as the pivotal mechanism for aging initiation and progression. In fact, in vertebrates, extension of lifespan and retardation of aging was achieved by reducing oxidative stress through overexpression of human catalase in mice mitochondria

(Schriner et al., 2005), and by mutating the mouse p66shc adaptor protein gene (Migliaccio et al., 1999; Giorgio et al., 2005), which is resposible of ROS-dependent mitochondrial apoptosis.

Three anticonvulsant drugs: ethosuximide, trimethadione, and 3,3-diethyl-2-pyrrolidinone, increase nematodes lifespan and delayed aging possibly through an action directed on central and peripheral neural activity (Evason et al., 2005). This effect was however strictly dependent on culture conditions, in fact all effects were observed at 20 °C, but not at 15 °C, where only 4 mg/ml of ethosuximide increased lifespan. Moreover results also varied in relation to the food substrate on which worms were fed (Evason et al., 2005).

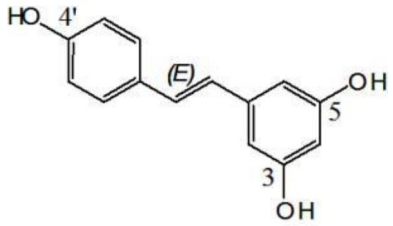

Figure 4.1: Resveratrol trans-isomer

Up to date, the only molecule which consistently prolonged lifespan across species is resveratrol (3,5,4'-trihydroxyl-trans-stilbene, Fig 4.1), a natural phytoalexin found in several plants where it plays its antifungal action (Hain et al., 1990; Langcake and Pryce, 1977). Notabily, resveratrol is present in the weed *Polygonum cuspidatum* and in the grape (*Vitis vinifera*), both being very important elements for human nutrition as basic food products in China-Japan and Mediterranean populations, respectively. Resveratrol is higly concentrated in red wine (5 mg/l in average, ranging from 0.5

to 10 ppm (Celotti et al., 1996)), which is considered the principal source of this compound for humans. This polyphenol has been shown to have many beneficial actions, ranging from anticancer (Jang et al., 1997; Manna et al., 2000), to anti-inflammatory, estrogenic, antioxidant and chemopreventive (Granados-Soto, 2003). Bioavailability of resveratrol is very low since, once consumed, it is very quickly absorbed (Bertelli et al., 1998) and 75% of it is lost by excretion via feces. The remaining part is mainly metabolized in glucuronide- and sulfate- resveratrol conjugates (Wenzel and Somoza, 2005), which are the biological active forms, since no aglycone resveratrol has been found after absorption (Goldber et al., 2003).[32] *In vitro* studies show that physiological concentrations of resveratrol modulate NF-kB (Nuclear Factor KB) dependent transcriptional activation in human endothelial cells (Pellegatta et al., 2003).

Recent studies have shown that resveratrol prolongs lifespan of yeasts (Howitz et al., 2003; Kaeberlein et al., 2005) and short lived invertebrates (Wood et al., 2004; Bauer et al., 2004; Viswanathan et al., 2005). The mechanims responsible for this reproducible effect on lifespan has been suggested to be the resveratrol-dependent activation of the NAD-dependent histone deacetylases belonging to the Sir2 family (Silent information regulator 2) (Howitz et al., 2003; Wood et al., 2004). Doubts have been casted on the *in vitro* resveratrol-dependent Sir2 activation in the yeast *Saccharomyces cerevisiae* (Kaeberlein et al., 2005; Borra et al., 2005). In vertebrates, resveratrol apparently activates the Sir2 homologue SIRT1 only in the presence of a fluorophore (Borra et al., 2005). Nonetheless, both in vertebrates and invertebrates resveratrol mimics the effects of SIRT1 overex-

[32] this raises some question mark on the in vitro studies using resveratrol in the aglycone form.

pression, inducing neuroprotection (Parker et al., 2005; Araki et al., 2004), and resveratrol effects require Sir2.1 (Wood et al., 2004). In the worm *C. elegans*, resveratrol prolongs lifespan through Sir-2.1, but independently from the forkhead transcription factor DAF-16 (Viswanathan et al., 2005). Interestingly, resveratrol administration further increases lifespan in Sir-2.1 overexpressing worms and two alternative pathways linking Sir-2.1 to longevity have been proposed, one dependent and the other independent from DAF-16 (Tissenbaum and Guarente, 2001; Tatar, 2005; Viswanathan et al., 2005).

Resveratrol has been suggested to mimic the effects of caloric restriction in yeasts, worms and flies (Howitz et al., 2003; Wood et al., 2004), but some doubts have been casted on this mechanism, at least in yeasts (Kaeberlein et al., 2005). A possible pathway linking resveratrol to caloric restriction and lifespan-increase can be dependent on the resveratrol induced upregulation of NO (Nitric Oxide), through the activation of NOS (NO synthase) (Imamura et al., 2002; Hattori et al., 2002). Endothelial NOS (eNOS) is induced by calorie restriction in many tissues of male mice and is responsible for many changes occurring with CR, like mitochondrial biogenesis, increased oxygen consumption, expression of 3'5' guanosine monophosphate and, importantly, SIRT1 upregulation (Nisoli et al., 2005). Very interestingly, resveratrol has been also shown to scavenge free oxygen radicals *in vitro*, particularly the superoxyde anion ($0_2{}^-$), and to displace ubiquinol, the reduced form of Coenzyme Q, which is the major ROS generator (Zini et al., 1999).

We have tested the effects of resveratrol on longevity and on age-related markers in the GRZ inbred *Nothobranchius furzeri* fed with this compound since the 4th week of life until death (Valenzano et al., 2006). For the first

time in a vertebrate, we have scored a significant dose-dependent increase in median and maximum lifespan after resveratrol food-administration (Fig 3.15). The lowest active dose was between 120μg/g (food) and 24μg/g (food), which correspond respectively to 0.5 μM and 0.1 μM. This dosage is remarkably lower than the dosage used for studies on invertebrates, which range from 10 to 500 μM (Wood et al., 2004; Bauer et al., 2004; Viswanathan et al., 2005). The effect was the same in the two sexes and resveratrol induced a slowing in the "rate of aging" measured as the slope of the death-rate plot (Fig 3.15C). The increased longevity in resveratrol-fed fishes was not due to a resveratrol-induced decreased feeding (i.e. unwanted dietary restriction) as fishes ate the whole amount of available food per day and also reached the same size of control-fed fishes at 9 weeks of age, implying that treated fish had a normal growth, despite the negative effects on size played by dietary restriction (Anson et al., 2003; Masoro et al., 1982). Resveratrol effect was not limited to a significant lifespan increase but also involved a rescue in locomotor and cognitive decay occurring in control fed fishes at 9 weeks of age, which were even more active than 5 weeks old fishes (Fig 3.16-18). In agreement with a resveratrol-dependent rescue in the cognitive decline, we also quantified a lowered age-dependent neurofibrillary degeneration in 9 weeks old treated fishes (Fig 3.19). We also found that, at 11 weeks, treated fishes, differently from control-fed, which were almost all dead and did not lay any egg, were still able to lay fertilized eggs. This can imply that resveratrol positive effect on lifespan is not dependent on a energetic investment on the soma at the expenses of reproduction, as *Disposable Soma Theory* of Aging would suggest (Kirkwood, 1977), but is apparently a pure fitness enhancer.[33] This problem raises the intriguing chance that a natu-

[33] the initial negative effect in survival deserves a further investigation.

ral resveratrol bioavailability would be a significant selective advantage in terms of fitness for a natural population over those that cannot access to this beneficial compound. It is interesting to note that, at least in two human distant cultures like mediterranean populations and China/Japan populations, a positive cultural selection on resveratrol rich plants-derivatives like wine and Kojo-kon (or Itari) has probably happened, possibly for the positive effects played by resveratrol on human health (i.e. darwinian fitness).

This study provides the first evidence that resveratrol can increase vertebrate lifespan and, also, that this compound protects *in vivo* from functional locomotor and cognitive decay, slowing down global aging. Our study confirms the proposed neuroprotective effect of resveratrol (Araki et al., 2004; Parker et al., 2005; Valenzano et al., 2006) and suggests also a possible role in retarding muscular aging. Whether resveratrol effects *in vivo* are due to its action on Sir2 family of histone deacetylases or to its antioxidant function, or both, is yet to be demonstrated. *Nothobranchius furzeri*, given its very short lifespan and its established age-dependent physiological decline, is an ideal animal model for the study of cellular pathways which are involved in longevity modulation, and is a very promising new model for assessing the role played by molecules like resveratrol and by other kind of interventions (like transgenic modulation of gene expression) in affecting these same pathways.

4.4 Aging in wild-derived *Nothobranchius furzeri* populations.

During a collection trip organized by Dr. Cellerino's team in Pisa, several wild *N. furzeri* populations were collected (Table 3.1). The collected populations showed a large variety in life exectancy which ranged from 9 weeks of median lifespan in MZM10pL to 23 weeks in MZM0403 (Fig. 3.21). Interestingly, this trend can be related to the environmental conditions in which these populations live, in fact, although all these populations were collected in an area of about 350km in diameter, the amount of precipitations within this area varies greatly and, in particular, longer living populations derive form more humid regions, whereas the short living MZM10pL was collected in a very dry area (around 325 mm of rain per year concentrated in 2-3 months), which is adjacent to the Gonarezhou National Park, where in 1968 Furzer and Warne sampled the first *Nothobranchius furzeri* population, by which the GRZ inbred line was thereafter derived (Jubb, 1971). This interesting trend which correlates average annual precipitations to longevity, recalls what has already been observed in different species of the genus *Nothobranchius*, where longer living species come from more humid regions compared to shorter living ones (Genade et al., 2005).

A comparison of death rate curves among different populations showed a very interesting trend, consisting in the same mortality rate in populations with different life expectancy (i.e. the linear interpolations are parallel), despite a strong variation for the timing of death rate increase, i.e. the differences among populations death rates concern the intercepts (Fig 3.21). This datum is coherent with a difference, among the various populations, in the onset of the death-rate increase. It would be worth studying the

timing of the gene-expression profile in natural populations which differ for longevity.

Since we developed a robust array of functional markers of senescence, we tested the wild-derived populations that we reproduced in the laboratory for locomotor activity and learning the *Active avoidance* task, to compare these results with those obtained in the GRZ inbred line. We observed an intersting dissociation between learning performance and locomotor activity decay, in fact, among the studied populations, only the GRZ inbred line showed a simultaneous cognitive and locomotor decay (Fig 3.24). Although the correlation between locomotor and cognitive performance in the GRZ inbred line could suggest a causal correlation among the two measures, possibly due to a reduced chance of learning in the less active fishes, all wild populations did not show any link about these measures. The long living MZM0403 population showed lower scores in learning the *Active Avoidance* taks at 5 weeks than other tested populations but, very interestingly, it showed a significantly increased locomotor activity from week 5 to week 9. Moreover, a further evidence against the causal correlation between locomotor activity and learning performance is the fact that MZM0403 population, which had the lowest scores in the *Active Avoidance* taks at 5 weeks, achieved at 9 weeks higher scores for motility than *good learning* 5 weeks old GRZ inbred fishes.

The important conclusions emerging from the study of wild-derived *N. furzeri* populations are the following:

- Short lifespan is not the effect of artificial selection but a natural trait of some populations of *Nothobranchius furzeri*.
- Natural populations are highly heterogeneous in longevity, as observed

in population MZM10 (Fig. 3.22).

- There is a dissociation between longevity and aging biomarkers, emerging from the fact that MZM10pL share with MZM10gr the same trend of age-associated behavioral changes (Fig. 3.24), but their longevity and mortality is significantly different (Fig. 3.21 and 3.23). On the other hand, MZM0403 and MZM10gr have small differences in longevity and mortality (Fig. 3.21 and 3.23), but large-scale differences in age-associated behavioral changes (Fig. 3.24).

- Very interestingly, we found that captive strains derived from localities facing higher extrinsic mortality rates due to pools desiccation (locality MZM10 vs. locality MZM0403, Fig. 3.20), face an age-related onset of behavioral senescence concerning cognitive performance in the *Active Avoidance* test (Fig. 3.24), but do not uniformely show higher mortality or decrease in exploration (*Open Field*-like test). This result recalls the association of higher extrinsic mortality rates with a "mosaic pattern" of senescence markers which was found in guppies (*Poecilia reticulata*) (Reznick et al., 2004), againsts the simpler prediction that high natural mortality would induce a generalized earlier onset of aging markers (Pleiotropic Antagonistic Theory, see *Introduction* chapter) (Williams, 1957).

The phenotypical differences found among *N. furzeri* wild populations are very likely due to a genetic basis, since all the populations were grown, fed and reproduced, in the same conditions (see *Methods* chapter). It will be of high interest to uncover which genes are responsible for these differences and to understand why natural populations of this group of Cyprinodontiform fish evolved this large variation for longevity. Since some of the mechanisms

that regulate lifespan in organims are widely shared from yeasts to rodents (Tatar et al., 2003), and very likely also for humans, it is to expect that findings on the genetic of fish longevity will be of interest for the human species as well.

We have not enough data to make conclusions about the genetic causes of the different longevity observed in short-lived and long-lived populations of *N. furzeri*, and whether these differences are in regulatory- or in coding-regions, but we can speculate that these observed differences in longevity could be due to variations in the levels of expression (regulatory regions?) of some of the genes involved in the major pathways known to modulate longevity in other model organisms (Insulin/IGF-1; FOXO family; Sir2 family), which could be good candidates to look at for starting a genetic analysis of the aging/longevity differences among wild populations of this species.

4.5 Hybrid-line generation

A preliminar experiment aimed at understanding the genetic of longevity in *N. furzeri* populations consisted in crossing the short lived GRZ inbred line with the longer lived population MZM03, which, among the populations we have studied, reaches the highest "maximum lifespan" (28 weeks). We crossed both male GRZ x female MZM03 and vice-versa. It was necessary to perform these two crossings in order to understand if the longevity trait was sex-linked or whether it was exclusively autosomic. Up to date we established the survival curves for the F1 generations of these two crossing experiments (complessively 55 fishes). Both crossings were significantly longer living than the short living GRZ inbred line, and the F1 generation derived from the MZM female x GRZ male also lived significantly longer than the F1 generation derived from the complementary crossing (Fig 3.25). Although we need more replicates to further confirm the differences among the two crosses, we observe that MZM03-longevity phenotype is dominant over the GRZ inbred-longevity phenotype. A more detailed analysis of the trait needs the study of the F2 generations, useful to assess whether the longevity trait in this species is dependent on one sinlge gene or, more likely, on more than one gene. It is also worth mentioning that the difference in longevity observed between the two crossings can be due to the fact that the MZM0403 wild-derived stock is expected to have a higher degree of polymorphism compared to the GRZ inbred line. For this reason, the differences in the survival curves of the two crossings might be due, more than to different patterns of inheritance, to differences in allele composition of the male and female MZM0403 used for making the crossings.

We were also interested in testing the inheritance of the behavioral age-

associated traits which were highly divergent among the two parental lines GRZ inbred and MZM03. The F1 hybrid generation obtained by crossing female MZM03 with male GRZ, underwent a significant cognitive decay from the 5th to the 9th week of age, resembling the trend shown by the GRZ inbred paternal line (Fig. 3.26A). Very interestingly, this F1 generation, unlike the paternal line, but like the maternal line, showed an increase in locomotor activity from the 5th to the 9th week of life. This datum supports the uncorrelation between the scores attained in the cognitive task with the measures of *Open Field* exploration, which are, evidently, under different genetic control and reveal, again (see previous section), a mosaic expression of aging traits (Reznick et al., 2004)

The development of a microsatellite linkage map would be pivotal for finding the genes involved in the regulation of longevity in different populations of *Nothobranchius furzeri*, as well as in different species of the genus *Nothobranchius*, following the approach developed by Kingsley lab at Stanford University (Peichel et al., 2001; Shapiro et al., 2004) on stickleback (*Gasterosteus aculeatus*). With such approach, it is possible to isolate the chromosomal region(s) which contain the microsatellites which are highly correlated with a given phenotypical trait (like longevity). In *Nothobranchius furzeri*, crossing a long-lived fish with a short-lived fish, this approach would allow to isolate the microsatellites which are statistically associated with the long(short) lived strain. The chromosomal position of the found microsatellites would help finding the gene(s) and the regions (regulatory or coding) responsible for the longevity phenotype.

4.6 Final considerations and future directions

In this thesis I showed and discussed the great potential for studies on longevity and aging offered by the Cyprinodont annual fish *Nothobranchius furzeri*. This verterbate model belongs to the class of short-living model organisms, together with widely used models like the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster*. Moreover, being a vertebrate, *Nothobranchius furzeri* is phylogenetically closely related to mammals, compared to worms and flies, therefore it could become a model of election for the study of vertebrates aging.

The last years assisted to an explosive development of fish-genomics, with the sequencing of five fish genomes (Zebrafish, Medaka, *Takifugu*, *Tetraodon* and Stickleback) and the development of transgenesis for commercial exploitation in salmon and rainbow trout (Clark, 2003). The study of fish models was also fruitful in terms of biological technical advancement since it led to the discovery of new tools for genetic engineering, like the transposons *Sleeping Beauty* (SB), found in the salmon (Ivics et al., 1997), and *Tol2*, found in Medaka (Kawakami et al., 2000), both successfully used in mammalian transgenesis (Miskey et al., 2005). The growing bulk of data regarding aging in *Nothobranchius furzeri*, together with the development of the above mentioned genomic tools in fish, provide a great starting point for developing *Nothobranchius furzeri* as a new competitive genetic model of aging. It would be of high theoretical and applicative importance to develop technologies wich adopt *Nothobranchius furzeri* for screening drugs that affect lifespan and for assessing the long-term effects on lifespan of genetic manipulations which modify metabolism, as well as to identify the reactions that mediate the effects of dietary restriction. As this thesis shows, *N. furz-*

eri can also be important for unveiling the role played by treatments like low-temperature, or compounds like resveratrol, in retarding the onset of the cognitive/locomotor abilities decay.

Appendix

Passive Avoidance task

I developed an original task for assessing measures of memory, therefore

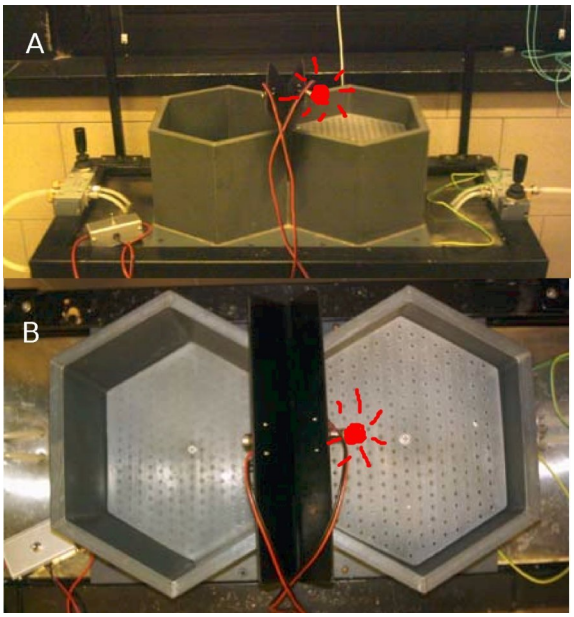

Figure 4.2: Passive Avoidance shuttlebox. The box is filled with water and the fish can pass from one compartment to the other. Holed platforms can raise, providing a brief shock to the fish, in the compartment where the red ligth is switched on (Conditioned Stimulus). (A) Lateral view. (B) View from above.

useful to test memory retention of a learned task. In order to do so, I used a

model similar to the shuttlebox described in the previous *Active Avoidance* task. Fig 4.3A shows a lateral view of the shuttlebox with vertical raising platforms that take the fish out of the water as non-conditioned stimulus. The rationale of the task is the following: to train the fish to avoid the compartment where the light is constantly "on". A red ligth is constantly "on" in one of the two compartments. In the same compartment where the light is "on", a finely holed platform gently raises every 30s for a duration of 5s. Fish can pass from one compartment to the other through a rectangular

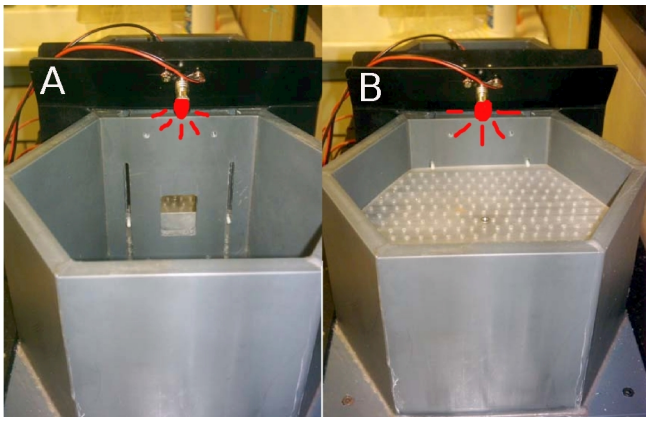

Figure 4.3: Passive Avoidance shuttlebox viewed from the inside. (A) when the platform is down, the fish is free to pass through the rectangular door connecting the two compartments. (B) The raised platform in the light compartment does not allow the fish to escape from the unconditioned stimulus (US).

door (4cm high x 3cm wide) only when both platforms are not raised (Fig A4-3A). Fishes must learn to avoid the ligth compartment. After completion of training (experimenter can choose to train fishes for several sessions also in consecutive days) the testing phase starts. The fish is put back in the compartment where the light (conditioned stimulus) is "on". Since

this point, for 5 min it is measured the time spent by the fish in the ligth compartment. This measure is compared to the same measure taken in a pre-training phase where only the conditioned stimulus is present. Briefly the test consist in the following phases:

- Pre-training phase (CS only), measure of preference for ligth or not-light environment (it is measured the percentual time spent in each compartment). Ten min duration. The fish is first placed in the ligth compartment.
- Training phase (CS and US), one or more sessions of 1 hr each.
- Testing phase (CS only), measure of latency to escape in the compartment where the light is not "on". Ten min duration. The fish is first placed in the ligth compartment.

The testing phase is determinant to assess the memory retention of the task, i.e. putting the fish back in the system one expects that, a fish which remembers the association of CS and US, would prefer to spend a significantly higher amount of time in the non-lighten environment compared to the score obtained in the pre-training phase. I have measured the natural preference of GRZ inbred and wild derived *Nothobranchius furzeri* for the ligthen vs. non ligthen compartment. The time spent in sample pre-training phase in the ligthen compartment is significantly higher ($\chi_2, N = 20, p < 0.001$) than that spent in the other compartment, i.e., differently from rodents, which prefer darker rooms (Selcher et al., 2001), this species has a natural preference for light.

With this new test it is possible to score memory performance at differ-

ent time-intervals from the training, in order to score age dependent memory decay but, also, treatment dependent memory enhancement/inhibition. With this same test it is also possible to separate the effects of short term memory from those of long term memory.

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Acknowledgments

(Ringraziamenti)

Il lavoro descritto in questa tesi é il frutto di tre intensi anni di dottorato presso il laboratorio di Neurobiologia della Scuola Normale Superiore di Pisa, dove ho avuto la fortuna ed il privilegio di perseguire fin dal primo anno un progetto di ricerca originale, insolito e scientificamente ambizioso che, dai corridoi dell'Istituto di Neurofisiologia del CNR di Pisa, mi ha portato fino alle strade sterrate nel cuore della savana africana, in Mozambico, tra baobab maestosi ed un paesaggio umano profondamente segnato da un lunghissimo conflitto ma appassionatamente proiettato verso un futuro di speranza.

Il mio percorso di ricerca ha seguito fin dall'inizio un tragitto irregolare, seguendo binari nuovi, non segnati da altri, ma tracciati dalla fiducia nelle nostre idee e dalla pragmatica pianificazione del piccolo gruppo di cui sono fiero di essere stato un elemento, e del cui capitano parlerò in seguito.

Il lavoro che ho svolto a Pisa sarebbe stato impensabile e non affatto possibile in assenza delle persone con cui ho lavorato in questi tre anni in questo parco della ricerca situato nel cuore dell'Italia.

Sento di ringraziare il professor Lamberto Maffei, per avermi dato la possibilità di lavorare al mio "insolito" progetto, e per avere trasformato la sua perplessità e stupore iniziale nei confronti di questo in incoraggiamento sincero e concreto appoggio scientifico.

Sono profondamente riconoscente nei confronti del professor Luciano Domenici, per la sua preziosa attitutine al dialogo informale e per il suo acume critico, nonché per la sua aperta disponibilità nel con-

cedermi spazio e fiducia.

Ringrazio il dottor Yuri Bozzi, il dottor Matteo Caleo e la dottoressa Laura Gianfranceschi, per essere stati per me degli interlocutori franchi e diretti, nonché dispensori di preziosi consigli, di cui faccio geloso tesoro.

Ringrazio anche il professor Tommaso Pizzorusso, la dottoressa Elena Putignano ed il dottor Alessandro Viegi, per la sincera cordialità riservata nei miei confronti in questi anni.

La mia esperienza di vita e ricerca a Pisa é stata fin dal primo anno segnata dalla fatale conoscenza del dottor Alessandro Sale, mio amico fraterno ed interlocutore d'elezione per quei momenti esaltanti di slancio intellettuale, in cui ci si spinge con coraggio a svelare le trame segrete dei massimi sistemi, e da cui si torna invariabilmente più ricchi ed intellettualmente vicini.

Sono riconoscente nei confronti del dottor Nicola Origlia, compagno di laboratorio ed amico, per la sua umiltà scientifica e per il suo sarcasmo, che mi hanno insegnato a riflettere sulla natura esile delle verità della scienza.

Ringrazio Giulio Cesare Cappagli e Carlo Orsini, per la infinita pazienza e disponibilità mostrata nei miei confronti e per aver saputo risolvere mille problemi tecnici; ed anche Claudio Palla, per aver avuto il ruolo di dare forma concreta ai miei apparati per la misurazione dell'apprendimento dei pesci.

Ringrazio gli amici con cui ho condiviso il laboratorio in questi anni: Laura Costantin, Silvia Landi, Matilde Marchi, Francesca Ciucci, Flavia Antonucci, Elisa Brilli, Jose Fernando Maja Vetencourt, Vincenzo Pignatelli, e molti altri, con cui ho passato bellissimi momenti di confronto scientifico e reciproco supporto.

Ringrazio Eva Terzibasi, Martina Calamusa, Tyrone Genade e Giandonato Tartarelli, con cui ho lavorato direttamente, per avermi trasmesso la loro passione per la ricerca e per avermi concesso la loro amicizia; Stefano Valdesalici e Miles Parisi, che, pur non biologi, mi hanno trasmesso tutta la traboccante voglia di conoscere che solo un vero appassionato può avere.

La persona che più di tutte ha pesato sulla realizzazione di questa tesi e a cui devo tutta la mia riconoscenza é senza dubbio alcuno il dottor Alessandro Cellerino, mio maestro ed amico, che ha dato forma e direzione al progetto su cui ho lavorato in questi anni, e che é stato colui

che ha avuto la geniale idea di usare *Nothobranchius furzeri* come modello per lo studio dell'invecchiamento. Dal dottor Alessandro Cellerino ho appreso moltissimo, imparando che il dato é piú forte della teoria (*"...data are data!"*), che la propria originalità scientifica va rispettata e protetta, e con essa le proprie idee, e che non bisogna temere di affrontare nuovi campi di ricerca, purché si sia mossi da un'autentica passione e disposizione allo studio.

Non posso non essere riconoscente nei confronti della mia meravigliosa famiglia, i miei genitori, i miei fratelli Layla ed Emanuele e le mie nipotine Erika ed Elena, che mi hanno sempre amato e che hanno sempre avuto fiducia in me. Un ringraziamento particolare lo devo ai miei genitori, che, come solo i migliori genitori sanno fare, hanno saputo darmi l'opportunità di scegliere in libertà la mia strada.

Infine dedico questa tesi alla mia nuova piccola grande famiglia: mia moglie Francesca ed il mio splendido figlio Nicola, che hanno riempito fin dal primo anno di dottorato la mia vita con la loro, in maniera imperiosa, prepotente, e che hanno reso tutto più bello, ogni conquista più sudata ed appagante, ogni momento autentico e sensato perché condiviso.