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REVIEW

Annual fishes of the genus *Nothobranchius* as a model system for aging research

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Summary

Aging research in vertebrates is hampered by the lack of short-lived models. Annual fishes of the genus *Nothobranchius* live in East African seasonal ponds. Their life expectancy in the wild is limited by the duration of the wet season and their lifespan in captivity is also short. *Nothobranchius* are popular aquarium fishes and many different species are kept as captive strains, providing rich material for comparative studies. The present paper aims at reviving the interest in these fishes by reporting that: (1) *Nothobranchius* can be cultured, and their eggs stored dry at room temperature for months or years, offering inexpensive methods of embryo storage; (2) *Nothobranchius* show accelerated growth and expression of aging biomarkers at the level of histology and behaviour; (3) the species *Nothobranchius furzeri* has a maximum lifespan of only 3 months and offers the possibility to perform investigations thus far unthinkable in a vertebrate, such as drug screening with life-long pharmacological treatments and experimental evolution; (4) when the lifespan of different species is compared, a general correlation is found between wet season duration in their natural habitat and longevity in captivity; and (5) vertebrate aging-related genes, such as *p66Shc* and *MTP*, can be easily isolated in *Nothobranchius* by homology cloning. These fishes can become excellent models for aging studies. They can be employed to test the effects of experimental manipulation on aging at a pace comparable with that of *Drosophila* and to probe the effects of natural selection on the evolution of aging-related genes.

Key words: aging; biomarkers; comparative genomics; *Nothobranchius*.

Introduction

Annual fishes are a group of teleosts that successfully colonized seasonal bodies of water by producing desiccation-resistant eggs which survive the dry season (Jubb, 1981). Their life expectancy in the wild cannot exceed the seasonal duration of their habitat and their extrinsic mortality probability is basically rectangular. They therefore represent an excellent model to study the effects of extrinsic mortality rate on the evolution of senescence.

American annual fishes of the genus *Cynolebias* (*now Austrolebias*) were studied by Walford and colleagues in the 1960s and 1970s (Walford *et al*., 1969; Liu & Walford, 1970; Liu *et al*., 1975). The African annual fish *Nothobranchius guentheri* was studied by Markofsky and colleagues in the 1970s (Markofsky & Perlmutter, 1972; Markofsky, 1976; Markofsky & Matias, 1977; Markofsky & Milstoc, 1979a,b). These researchers reported that annual fishes have a defined lifespan in captivity that can be modulated by water temperature and that they develop age-dependent histological degenerations in a wide array of organs such as the eye, liver, kidney and thymus. These results, combined with the work of Comfort and Woodhead in the guppy *Poecilia reticulata* (Woodhead, 1998), demonstrated that teleosts are pertinent models for vertebrate aging.

The scientific community remained unimpressed by this remarkable body of evidence and the fishes never gained popularity in gerontology. Only recently, riding the upsurge of interest for zebrafish genomics, some papers on fish aging have reappeared (Gerhard *et al*., 2002; Kishi *et al*., 2003; Murtha & Keller, 2003; Herrera & Jagadeeswaran, 2004; Müller, 2005).

Annual fishes are not only exotic animal models, however, they are also among the most colourful and sought-after aquarium fishes. Although the scientific community was agnostic as to their potential, a handful of dedicated hobbyists over the last 30 years have systematically sampled eastern Africa looking for new species of *Nothobranchius*. As a consequence, detailed knowledge of their systematics, distribution and habitat is available (Wildekamp, 2004).

More importantly, the primary aim of these expeditions was to obtain breeding stock and to establish captive breeding colonies. As *Nothobranchius* eggs can be easily shipped by ordinary post, after each collection trip, the new populations were rapidly distributed to a tight-knit community of hobbyists around the world to secure their preservation in captivity. Almost a hundred different populations of *Nothobranchius* have been maintained without hybridization (Seegers, 1997; Wildekamp, 2004), and many are still extant as inbred captive strains. For this reason, *Nothobranchius* fishes not only represent convenient aging

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models due to their rapid senescence, but offer an unprecedented opportunity for comparative studies.

The aim of the present review is to put *Nothobranchius* fishes under the spotlight of the aging research community to reveal their long overlooked potential as model organisms.

Much of the information we will report here does not stem from the work of professional researchers, but from the hobbyist literature or personal communications from those who collect, breed, distribute, maintain, observe and enjoy these colourful fishes. It should be noted that many of these dedicated and famous hobbyists, who are referenced here (Foersch, Huber, Jubb, Nielsen, Scheel, Seegers, Shidlovsky, Watters and Wildekamp) are/were themselves scientists, and mostly biologists, with some stature in their respective fields. Their knowledge of *Nothobranchius* biology is better than anyone else, having dutifully observed them in the wild and over many generations in captivity. This information is anecdotal and not substantiated by data collected with scientifically acceptable protocols. However, we feel that it provides invaluable hints for future research on *Nothobranchius* biology.

Biological history

Nothobranchius is a genus of the family Cyprinodontiformes, closely related to the pupfish *Cyprinodon* species and the mumichog *Fundulus heteroclitus* of North America, and to the guppy *Poecilia reticulata* (Wildekamp, 2004; Huber, 2005). The genus comprises 43 described and several undescribed species (Seegers, 1997; Wildekamp, 2004). These animals have a marked sexual dichromatism. Females of different species can hardly be distinguished morphologically and the classification is based mainly on male livery and size (Scheel, 1968; Jubb, 1981). They are relatively small fishes with a median size of 5 cm (ranging from 4 to 15 cm). These fish are all adapted to survive in temporary waters and all produce eggs which not only resist desiccation but require a dry period to develop correctly, at least in captivity.

Habitat and distribution

Nothobranchius range from Kwa-Zulu Natal, South Africa, in the south (Wildekamp, 2004) to Angarko, Sudan, in the north (Bellemans, 2003). Zanzibar, Tanzania, is the eastern boundary of their distribution and Lake Chad the western boundary (Watters, 1998). Only three species occur off mainland Africa on islands (Zanzibar and Mafia Island) that were previously connected via a land bridge to Tanzania (Wildekamp, 2004).

They inhabit ephemeral pools (typically on floodplains) that may, during heavy wet seasons, be temporarily connected to rivers and streams (Jubb, 1981; Seegers, 1997; Watters, 1998; Wildekamp, 2004). All known habitats have a substrate composed of vertisols developed over alluvial deposits (B. Watters, Department of Geology, University of Regina, Canada, cited in Wildekamp, 2004), which is suggested to be crucial in creating the microhabitat for long-term egg survival.

The water quality in *Nothobranchius* habitats is often not pristine. It is reported that the Sazale pan in Zimbabwe was full of elephant faeces when *N. furzeri* was captured (Jubb, 1972); and pools in Tanzania where *N. eggersi* were collected in large numbers were polluted by cow dung (S. Valdesalici, Associazione Italiana Killifish, Viano, Italy). Daily variations in temperature can be extreme, especially in the highland habitat where oscillations of more than 10 °C are not uncommon.

Evolutionary biology

Molecular cladistic analysis indicates that the genus is monophyletic and that species diversity is due to vicariance (Murphy & Collier, 1997, 1998). The data suggest that the only two species closely related to *Nothobranchius* are *Fundulosoma thierryi* and *Pronothobranchius kiyawensis*. These species are morphologically similar to *Nothobranchius* and show the same annual life-cycle, but are found to the west of Lake Chad (Scheel, 1968). These three genera represent a distinct clade relative to other, non-annual, African Cyprinodontiformes (Murphy & Collier, 1998). Annualism is suggested to be a selective advantage that enabled these fish to exploit a unique niche and, in doing so, to escape competition with other teleost species (Seegers, 1997; Murphy & Collier, 1998; Hrbek & Larson, 1999).

Interestingly, the only other teleost species to be found with *Nothobranchius* (*unless* the pool is invaded by fish from an adjacent river) are the lungfishes *Protopterus*, which are able to aestivate in a cocoon during the dry season (Seegers, 1997; Hrbek & Larson, 1999). Lungfish have developed opposite adaptations to *Nothobranchius*: the adults and not the embryos are able to survive dormant in the dry mud. As a consequence, lungfishes are very long-lived fish with captive records of more than 20 years.

Biodiversity of *Nothobranchius* shows marked regional variations. Tanzania is an epicentre of a relatively recent *Nothobranchius* radiation with no less than 16 clearly differentiated species being found within a 200-km radius of Dar es Salaam (Seegers, 1997), most of them with very restricted distributions. On the other side of the spectrum, *N. orthonotus* and *N. rachovii* colonize an area of more than 2000 km including the Kruger National Park in South Africa, the coast of Mozambique up to the Zambezi River and southern Malawi (Wildekamp, 2004).

Remarkable variations in regional climate and precipitation patterns are observed in *Nothobranchius* habitats. Some (e.g. Zanzibar) have two short dry seasons which punctuate the long wet season (Wildekamp, 2004), while other localities may not dry out at all in some years (e.g. Mafia Island) (Seegers, 1997). By contrast, some of the locations where *Nothobranchius* are found, such as Sudan or the Kruger National Park, show brief and erratic rainy seasons and may not receive sufficient rain for several years (Jubb, 1981). As discussed in more detail below, differences in precipitation patterns result in large-scale differences in captive longevity for different *Nothobranchius* species.

South American annual fishes, by contrast, all reside in relatively humid habitats. They live longer than their African counterparts and some can reach 36 months if kept at temperatures compatible with those encountered in their natural habitat (Liu *et al.*, 1975).

Reproductive and developmental biology

The fish of this genus have adapted to the routine drying of their environment by evolving desiccation-resistant eggs that can lie dormant in the mud for one or perhaps more years (Watters, 1996; Wildekamp, 2004).

This delay in development is accomplished by the eggs going into diapause (Jubb, 1981). Under laboratory conditions, diapause is characterized by three stages (Wourms, 1972a,b,c). In DI (the first diapause) blastogenesis has completed and embryogenesis has not yet begun. At DII embryogenesis is complete and organogenesis has not begun. At DIII organogenesis is complete and the fully formed fry awaits the environmental cue to hatch.

The regulatory mechanisms of the diapauses are not well understood and are reviewed by Kadlec & Vitek (2003).

It is hypothesized from various researchers (e.g. Wourms, 1972c) and hobbyist observations that the eggs develop in a staggered fashion over time. Bellemans (2000) investigated the status of eggs in mud samples collected in Sudan 3 months after the respective pan dried out and reports finding only eggs in DI. The eggs can remain in DI for a very long time, at least under captive conditions.

Eggs spawned in captivity of *N. rachovii* from the Kruger National Park have remained undeveloped for at least 5 years and *N. orthonotus* from the same locality for 4.5 years (B. Watters, personal communication). Two of the authors (Al.C. and T.G) have recently inspected a sample of eggs from *N. rachovii* which were 14 months old and the large majority of the eggs were still in DI. Very long diapause is also reported for eggs of *N. eggersi*, *N. furzeri*, *N. kafuensis* and *N. orthonotus*. These species (except for *N. eggersi*) originate from habitats with well-separated wet and dry seasons.

Newly hatched fry measure 5–8 mm depending on the species. As expected from animals with an accelerated life-cycle, *Nothobranchius* are famous for their explosive growth. Under captive conditions, if proper feeding and water quality are provided, most species can reach sexual maturity in 6 weeks and attain adult size in 2–3 months.

Growth in the wild may possibly be faster. In the Kruger National Park almost full-grown *N. orthonotus* (5 cm) were collected 5 weeks after their pool received the first rain (B. Watters, personal communication).

Nothobranchius as a laboratory animal

Captive care

Nothobranchius males are extremely colourful and are sought-after aquarium fishes. Captive care of *Nothobranchius* does not require any special technical equipment: they originate from small-sized closed pools and, in captivity, are tolerant of a wide array of water parameters, resistant to poor water quality and tolerant of wide temperature ranges (Watters, 1998). Although water chemistry is not a major issue, their captive care requires special attention to feeding and breeding. Culture conditions are radically different from those used for zebrafish, and zebrafish experts may fail in raising and breeding *Nothobranchius*.

In contrast to zebrafish, *Nothobranchius* originate from stagnant water. Zebrafish are usually kept in recirculating systems with strong water flow. An attempt to keep *N. rachovii* in a recirculating zebrafish set-up resulted in massive death of the fishes within less than 2 months (Herrera & Jagadeeswaran, 2004). The cause of this pathology needs to be investigated but it is possibly due to exhaustion, as *Nothobranchius* are not active swimmers and cannot swim against a constant current. On the other hand, *Nothobranchius* can be kept in tanks without any filtration, provided that at least 50% of the water is changed every week (Markofsky & Perlmutter, 1972; Valdesalici & Cellerino, 2003; Herrera & Jagadeeswaran, 2004). Hobbyists generally keep *Nothobranchius* in small (10–40 L) tanks with sponge filters driven by an air supply which provides gentle filtration. The survival of *N. furzeri* does not change if they are kept in stagnant water or with air-driven gentle filtration (Fig. 1).

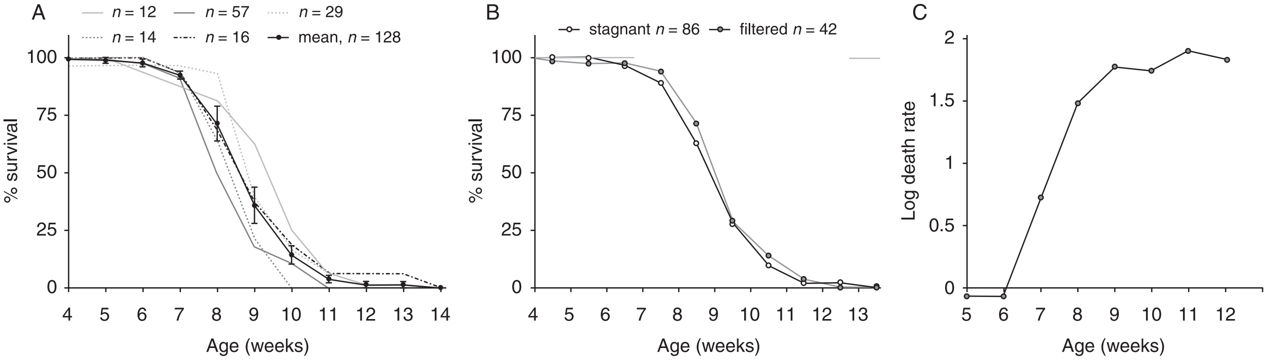

Fig. 1 Survival curves of *Nothobranchius furzeri* (Gona-Re-Zhou strain) maintained in the laboratory. (A) Normative data at 25 °C with two feedings per day. The thin lines are representative of five separate experiments; the solid line is the mean of all experiments. Note the reproducibility of the curves and the steep decline of survival between weeks 8 and 10. (B) Same data re-plotted separating the experiments performed in stagnant water (open circles) or with gentle filtration (solid dots). (C) Age-dependent mortality rate for the data presented in A.

The use of separated tanks is an advantage if chronic pharmacological experiments have to be performed because excretion products from different experimental groups remain separated.

Another major difference with zebrafish is male aggression: *Nothobranchius* males often display and attack other males and can attack females unwilling to spawn. Aggression of the dominant male is greatly reduced if fishes are kept in large groups. Ten to 20 fishes of balanced sex ratio in a 40-L tank is a good population for medium-sized species (*N. furzeri*, *N. guntheri*, *N. rachovii*). With a turnover of 3 months, up to 80 *N. furzeri* can be studied in one single 40-L tank in one year. A cupboard-sized set-up composed of 12 40-L tanks can house up to 1000 *N. furzeri* in one year.

The first critical point in *Nothobranchius* care is to provide them with quantitatively and qualitatively appropriate food during their rapid development. Unlike zebrafish, they need a high-protein diet able to sustain their explosive growth. Failure to do so results in permanently stunted growth and reduced female fecundity. Fishes raised by hobbyists rarely reach the size of wild-caught specimens unless large quantities of live and frozen food are provided. Our experience with *Nothobranchius furzeri* is that feeding with newly hatched brine shrimps, the standard food for fish *larvae*, does not support their growth well. Brine shrimps need to be fortified with commercially available food supplements rich in highly unsaturated fatty acids. The larvae then need to be 'weaned' as soon as possible on finely chopped food high in protein content. The most practicable option is commercially available frozen *Chironomus larvae* (bloodworms). Bloodworms can be administered as the only food for the remainder of their lives. Fishes then need to be fed 2–3 times a day all the food they are able to eat in 5 min. A less laborious alternative is to feed large amounts of freshwater live food (*Chironomus* sp., *Tubifex* sp., *Lumbricus variegatus*) which survive for days in the tank. Regular water changes (ranging from 20% daily to 50% bi-weekly) are needed to remove the wastes produced by the very large amount of food needed.

The second critical point in breeding *Nothobranchius* is the spawning substrate. In principle, any soft substrate wherein eggs can be deposited is accepted (peat, sand, glass beads, coconut fibres, etc.). For convenience, the substrate is placed in a small flat vessel that can be easily removed. The dominant male usually takes control of this vessel and chases away any other male. Hobbyists use boiled peat-moss and regularly remove the peat-moss containing the eggs; we prefer to use very fine sand, sift the eggs out of the sand, pick up the eggs and place them over damp peat-moss. If properly fed, the fish can be highly productive, with a female laying several hundreds or thousands of eggs during her lifetime (Seegers, 1997). However, the eggs are not released in one single spawn but a small number of eggs (20–30) are released every day. To increase egg production it is advisable to keep males and females separated and then place them together for a short time (1–2 days).

The third critical point is egg storage. Fresh eggs are large (about 1 mm in diameter or more, depending on the species), sturdy (can be safely picked up with blunt forceps) and can be stored in damp peat-moss for months. Peat-moss is the substratum of choice because of its ability to retain moisture. Too dry or too wet a substratum will both result in egg death. Hobbyists use the term 'tobacco consistency' to describe the correct dampness. Moreover, not all peat-moss types can be used and several should be tested to determine the best brand. Hobbyists place the peat-moss with the eggs in a sealed bag for months before hatching; we prefer to place the eggs over damp peat-moss in a sealed Petri dish because this allows continuous visual control over egg development. A 50% egg loss is normal under these storage conditions. Within a few days after being placed on damp peat, the chorion of the eggs hardens to the point that they bounce if accidentally dropped.

The speed of egg development strongly depends on environmental factors. Many of these factors, which have been scientifically investigated, are reviewed by Kadlec & Vitek (2003). Peters (1963) hypothesized that oxygen deprivation (in anoxic mud or peat) triggers DI and DII; and Watters (1996) predicted that those eggs not exposed to such conditions would develop normally without entering DI and DII. We (Al.C., T.G) have observed that *N. furzeri* eggs not exposed to oxygen deprivation do not enter DI or DII but develop to DIII in 3 weeks as opposed to 6 months. Similar observations are reported from the aquarium literature (Foersch, 1975).

Anecdotal evidence from killifish hobbyists, our unpublished observations and experimental evidence from Markofsky & Matias (1977) indicate that changes in temperature can extend, shorten or completely abolish DI and DII. As a consequence, the developmental time of eggs can be manipulated to be a few weeks or several months (for some species up to years). This offers great benefits in experimental planning and inexpensive methods of embryo storage.

The last critical point of *Nothobranchius* as laboratory animals is determining the right hatching window. If eggs are wet too soon, they do not hatch and may die. If eggs are hatched too late, the embryos are very weak and do not survive, or present anomalies in the swim bladder and develop into 'belly sliders'. The situation is complicated by the fact that the eggs rarely develop in synchrony. Some can be already in DIII while others are in still in DI. For these reasons, we find it useful to incubate eggs on top of damp peat-moss, so that developed eggs can be picked up and hatched while the others are left to develop. Eggs ready to hatch are recognized by the gold rim of the embryo's eye.

Hatching is stimulated by cold (18 °C) water and needs the presence of peat or peat extract. Water level needs to be very low (1 cm) so that the fry can swim up to fill their swim bladders. If eggs are hatched at the right time, the fry are quite robust and more than 80% can be expected to reach adulthood (Valdesalici & Cellerino, 2003; Herrera & Jagadeeswaran, 2004).

In summary, the husbandry and breeding of *Nothobranchius* requires little technical equipment but some finesse and experience to master the feeding, storage of eggs and timing of hatching. For those interested in establishing colonies of

Nothobranchius, we advise beginning with *N. guntheri* because of their long lifespan and short incubation times.

Nothobranchius are promising subjects to study the effects of environmental manipulations and/or pharmacological treatments on longevity. However, more work is needed for the development of artificial diets and methods for high-density, large-scale cultures which are necessary prerequisites for saturation mutagenesis, RNA interference and transgenesis.

Aging phenotype of *Nothobranchius*

Age-dependent survival trajectories are available for *N. guentheri*, *N. rachovii* and *N. furzeri*. Median survival in the three species is 12 months (Markofsky & Perlmutter, 1972), 6 months (Herrera & Jagadeeswaran, 2004) and 9 weeks (Valdesalici & Cellerino, 2003), respectively. *N. rachovii* shows a gradual, almost linear decline in survival. By contrast, the survival curve of *N. furzeri* is rectangular and shows an exponential increase in probability of death with age with a subsequent deceleration (Fig. 1). This temporal profile, observed in many different models as well as in humans, is considered a hallmark of age-dependent physiological deterioration and provides evidence that short lifespan in *Nothobranchius* is associated with rapid aging.

Histological analysis of aging was performed in *N. guentheri* by Markofsky & Milstoc (1979b). They detected marked aging-associated degeneration of the kidney (Markofsky & Milstoc, 1979b; Cooper *et al*., 1983) and liver (Markofsky & Milstoc, 1979a) as well as increased incidence of tumours (Markofsky & Milstoc, 1979b; Cooper *et al*., 1983). These observations were tied with a degeneration of the thymus, suggesting immune system decline with aging. Age-dependent degeneration of the pituitary was reported in *N. korthausae* (Ruijter *et al*., 1987).

At a macroscopic level, *Nothobranchius* fishes undergo progressive and apparent age-dependent degenerations, which are well known to hobbyists. Many breeders sacrifice the fish once the symptoms have developed, as old fish have a generally wasted appearance. Their stocky bodies progressively lose weight and appear thinner (Fig. 2). The females lose their rotund appearance taking on a look of emaciation and a curved spine. Calorimetric data show that this emaciation is associated with a progressive loss of fat (Markofsky, 1976; Balmer, 1982). Spinal curvature and emaciation are associated with aging in several other fish models, e.g. guppy, *Cynolebias* and zebrafish (Gerhard *et al*., 2002). In addition to these changes, males progressively lose their bright colours. In all *Nothobranchius* species we have had the opportunity to observe (*N. eggersi*, *N. guentheri*, *N. korthausae*, *N. kunthae*, *N. neumanni*, *N. orthonotus* and *N. virgatus*), but not in the extremely short-lived *N. furzeri*, these degenerations can be detected long before the death of the fish. It remains to be elucidated whether these changes are a primary consequence of a progressive failure of cellular and organ maintenance or rather the results of infectious diseases due to age-dependent degeneration of the thymus (Cooper *et al*., 1983).

Age-dependent reduction of male and female fertility under laboratory conditions is reported for the American annual fish *Austrofundulus limnaeus* (Podrabsky, 1999). Many hobbyists report age-dependent reduction in fertility and fecundity for *Nothobranchius* species. These anecdotal reports might be highly relevant because reduced fertility is a crucial parameter for age-dependent decay in fitness. A quantitative analysis of fertility and fecundity in different species is necessary to validate these suggestions and is a priority for future *Nothobranchius* research.

Ongoing research in our laboratory is focused on *N. furzeri*. Here we report a first analysis of expression of aging biomarkers. Histological investigations have shown increased levels of fluorescent age pigment (lipofuscin) in the liver of 9-week-old *N. furzeri* when confronted with 5-week-old fishes (Fig. 3A,B). Expression of senescence-associated β-galactosidase, a putative marker of replicative senescence (Dimri *et al*., 1995), observed during zebrafish aging (Kishi *et al*., 2003), was also detected in dermis fibroblasts of aged *N. furzeri* (Fig. 3C,D). Behavioural and video-tracking analysis has shown reduced spontaneous movements with advancing age (Fig. 4A) and a decline in open-field exploration (Fig. 4B). All these observations indicate that even this extremely short-lived species shows expression of cellular biomarkers of aging and undergoes age-related physiological decay.

N. furzeri has a maximal lifespan of only 3 months, similar to that of wild-derived *Drosophila*, and can offer the possibility to perform investigations so far unthinkable in a vertebrate, such as drug screening with life-long pharmacological treatments (Kang *et al*., 2002; Wood *et al*., 2004; Evason *et al*., 2005) or experimental evolution (Stearns *et al*., 2000; Rose & Charlesworth, 1980; Rose *et al*., 1992).

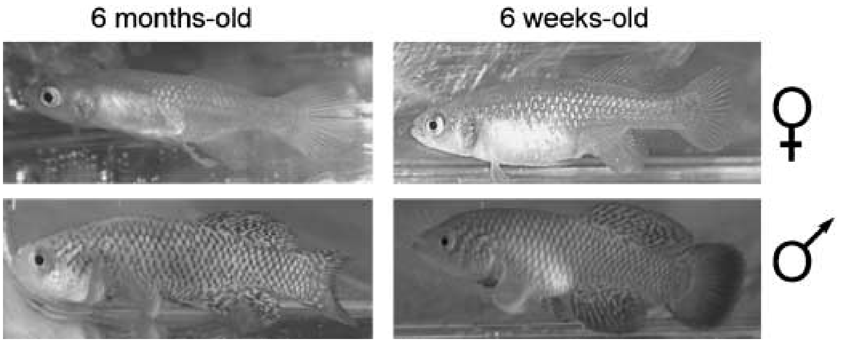

Fig. 2 Male and female *Nothobranchius* (undescribed species from the lower Limpopo River) at 6 weeks and 6 months. Note emaciated state of old fish.

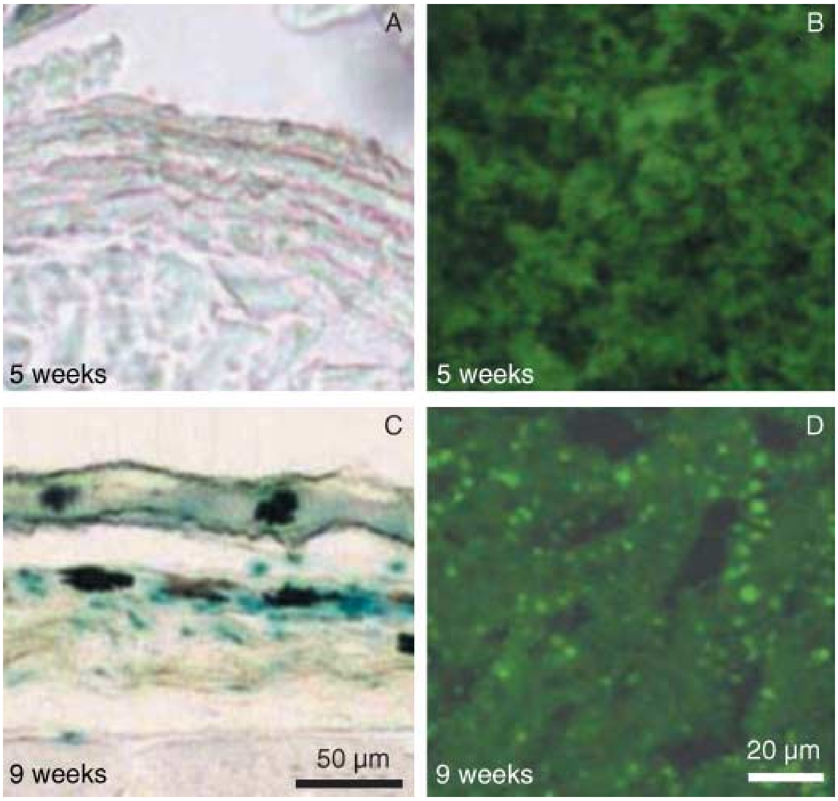

Fig. 3 Histological markers of aging in *N. furzeri*. (A) Coronal section of the skin and dermis in 5-week-old fish. Staining for senescence-associated β-galactosidase according to Kishi *et al*. (2003). Note absence of specific staining. Black cells are melanophores. (B) Liver section from a 9-week-old fish. Lipofuscin autofluorescence detected under 488 nm excitation with a confocal microscope. (C) Coronal section of skin and dermis. Cells labelled by specific histochemical reaction are blue. (D) Liver section. Laser intensity, photomultiplier and pinhole settings as in B. Note the abundant presence of autofluorescent globular structures.

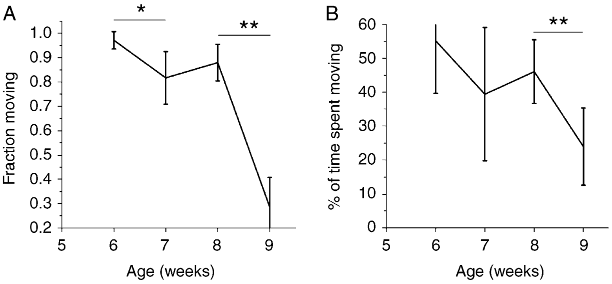

Fig. 4 Age-related locomotor deficits of *N. furzeri*. (A) Spontanteous locomotion. Animals were videotaped in their home tank. The average proportion of animals actively swimming during a 5-s clip is reported. Each point is the average of ten measurements. (B) Open field exploration. Animals were singly transferred to a novel tank and videotaped for 5 min. Clips were analysed using Ethovison® (Noldus, The Netherlands) and the fraction of time spent moving was quantified for each fish. Measures are from means of ten animals. Error bars are standard deviations. Pair-wise comparisons were performed using Kruskall–Wallis rank-based ANOVA; $*P < 0.05$, $**P < 0.01$.

An open question of *Nothobranchius* aging is the relationship between incubation time (as well as incubation protocols) and longevity. As we have previously mentioned, egg development is not synchronous and some eggs develop more rapidly than others even in highly inbred strains such as *N. furzeri* and *N. rachovii*. Of even more importance is the relationship between diapause length and longevity in wild-derived strains. We are currently addressing this issue by analysing the F2 generation of a wild-derived population.

Comparative aspects of aging *Nothobranchius*

Thirty-four of the 43 recognized *Nothobranchius* species, in addition to many undescribed species, are maintained by killifish hobbyists around the world. Many of these species are represented by several distinct populations (Seegers, 1997; Wildekamp, 2004). The most common of these different species and strains are not difficult to obtain via the various dedicated hobbyist organizations.

Nothobranchius have a long history in captivity; the first strain to be established was that of *N. rachovii* collected from the Beira region of Mozambique in 1958 (Wildekamp, 2004). Since then there have been numerous collections over a wide area not just for *N. rachovii* but also for other species. For many species, several populations, originating from different collections, are available.

The availability of different populations is a key advantage for comparative studies. Effective population size of *Nothobranchius* in the wild can be very limited due to the scattered nature of their habitat, which consists of isolated pools separated by dry land. Different populations can show profound founder effects and genetic drift evident by marked phenotypic differences.

A good measure of this isolation is the reproducible difference in colour patterns observed between geographically close populations of the same species; this is well illustrated in the aquarium literature (Seegers, 1997). An extreme example is the population of *N. eggersi* collected from the Rufiji River floodplain in Tanzania. In 1995, a population with a brilliant red body colour was collected (Sainthouse, 1996). Only 2 km from this locality, a population with a blue body colour was found. These populations are still kept by hobbyists under the collection codes TAN 95/7 and TAN 95/8 and the chromatic difference remains after many generations in captivity. A second expedition in 2002 confirmed the existence of two distinct colour morphs separated by only a few kilometres, and these populations (phenotypically indistinguishable from the 1995 collection) are currently bred under the collection codes TAN 02-12 and TAN 02-15. The phenotypic difference indicates that a genetic barrier must exist between these two populations, although this needs to be quantified by analysis of DNA polymorphisms.

The example of *N. eggersi* was described in some detail because it is representative of several *Nothobranchius* species. Multiple collections have targeted the same locality, each giving rise to different captive strains, uniquely recognized by a specific collection code. Because hobbyists consider crossing of different populations anathema and long-term maintenance of pure strains a hobby mission, both highly inbred and wild-derived strains are available for the same species. For example, *N. rachovii*, the most popular *Nothobranchius* among hobbyists, was originally collected near the city of Beira in Mozambique in 1958 (Jubb, 1981). This population is still extant as a captive strain. The same species was then collected again in the same locality in 1991, 1998, 2003 and 2004, giving rise to four different wild-derived strains (Wildekamp, 2004).

Most interesting in this context is the effect of extrinsic mortality probability on the evolution of life-history traits in these fish. The three main theories of aging (antagonistic pleiotropism, accumulation of neutral mutations, disposable soma) (Medawar, 1952; Williams, 1957; Kirkwood & Holliday, 1979; Hughes & Reynolds, 2005) all predict that differences in extrinsic mortality rates result in differences in growth, which eventually cause differences in senescence. In particular, high mortality rates drive evolution of rapid growth and maturation and, as a secondary consequence, of rapid senescence. These predictions were confirmed by experimental evolution in *Drosophila* (Stearns *et al.*, 2000). However, there are only limited data as to the effects of different mortality rates on the evolution of life-history traits in natural populations of vertebrates. There are examples of negative correlations between mortality rates and longevity in natural populations of opossum and salmon (Austad, 1993; Hendry *et al.*, 2004). However, the most complete study of natural populations of a vertebrate, performed in the guppy (*Poecila reticulata*), does not support this theory. Guppies originating from high-predation sites (subject to high extrinsic mortality) show accelerated growth and early sexual maturation, but not reduced longevity or shortened reproductive lifespan (Reznick *et al.*, 2004).

For annual fishes, extrinsic death probability can be assumed to be rectangular and is determined by the duration of their habitat. Owing to the huge differences in extrinsic mortality rates over their distribution area, the ease by which extrinsic mortality rates can be estimated by meteorological records, and the large number of species for which captive colonies are available, *Nothobranchius* fishes are an exceptionally good model system to study the effects of extrinsic mortality rates on evolution of life-history traits.

Age-dependent survival in the laboratory has been recorded for only three species so far: *N. furzeri*, *N. rachovii* and *N. guentheri*. Maximal survival is 3 months (Valdesalici & Cellerino, 2003), 9.5 months (Herrera & Jagadeeswaran, 2004) and over 16 months (Markofsky & Perlmutter, 1972), respectively. A remarkable correlation between the rainfall pattern in the biotope of origin and captive lifespan exist for these three species. Home of *N. furzeri* is the low veld of Zimbabwe, which, apart from actual desert areas, is the driest region of the entire subcontinent, with annual rainfall in the order of 360 mm year^{-1} (data for Breitbridge, www.worldweather.com). *N. rachovii* originates from the coastal city of Beira, Mozambique, with a clear separation between wet and dry season, but with a more humid climate and average annual rainfall in the order of 1600 mm year^{-1} (www.worldweather.com). *N. guntheri* originates from Zanzibar, a humid mosaic forest with two wet seasons (www.worldweather.com). The habitats of *N. guentheri* might not dry every year. In fact, eggs of *N. guentheri*, in contrast to most other *Nothobranchius* species, can complete their development in water without the need for a dry period (Markofsky & Perlmutter, 1972).

To exclude the possibility that differences in captive longevity are due to different husbandry protocols across laboratories, we have studied the species *N. kunthae* from the city of Beira (where it lives syntopically with *N. rachovii*; see Jubb, 1981) and compared its survival with that for *N. furzeri*. The data are reported in Fig. 5(A). The survival of *N. kuntahe* in our set-up is similar to the survival of *N. rachovii* recorded by Herrera & Jagadeeswaran (2004). Median survival is 22 weeks and maximum survival is 38 weeks (data for *N. rachovii* are 24.5 and 39 weeks, respectively). Moreover, *N. kunthae* and *N. rachovii* show a gradual, almost linear decline in survival which contrasts with the more rectangular mortality curve observed in *N. furzeri*. These data provide evidence that differences in captive longevity between *Nothobranchius* species from arid and humid climates are not due to differences in culture conditions.

Growth curves for *N. furzeri* and *N. kunthae* are shown in Fig. 5(B). Representative specimens of the two species at age 5 weeks and at their maximum size are shown in Fig. 5(C–F). From these images it is clear that even if *N. kunthae* reaches a larger final size, growth of *N. furzeri* is faster. Moreover, *N. furzeri* males show adult coloration and breeding behaviour already at age 4 weeks whereas in *N. kunthae* this appears only at age 7 weeks. Short lifespan in *N. furzeri* is therefore tied with accelerated development.

These results call for a more extensive study of captive lifespan and life-history traits, including several species from both very humid and arid biotopes. *Nothobranchius* are a monophyletic group of morphologically very similar fishes. If the strong differences in senescence rates among species are confirmed, the genomes of these species could provide a rich data source for a comparative genetic analysis. It is important to stress that different lifespans in different *Nothobranchius* species are not

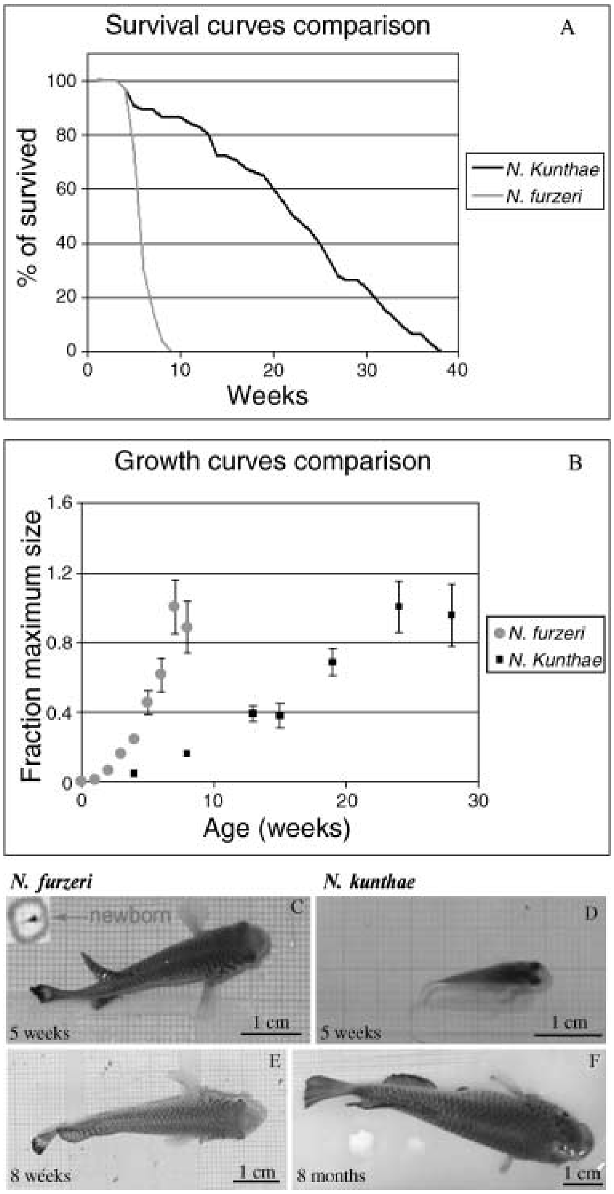

Fig. 5 Survival and growth of *N. furzeri* and *N. kunthae*. (A) Survival curves recorded under the same culture conditions. *N. furzeri* data are replotted from Fig. 1. *N. kunthae* data are representative of 65 fishes. (B) Normalized growth curves. Fish volumes were estimated from images (such as in C–F) using the formula of the ellipsoid volume $V = (2/3)\pi d^2 D$, were d is the width and D the length of the fish. Bars are standard deviations. (C) *N. furzeri* male at age 5 weeks. The small red circle encloses a newborn fry at the same scale. (D) *N. kunthae* male at age 5 weeks. Note the lack of nuptial livery, which indicates sexual immaturity. (E) *N. furzeri* male at age 9 weeks. Note the different scale with respect to C. (F) *N. kunthae* at age 8 months.

the result of random or targeted mutations as are those characterized in the current model organisms, but a secondary consequence of adaptations to radically different habitats. It is therefore unlikely that the phenotypic differences are due to polymorphisms of a single gene; they more likely depend on the action of molecular evolution on a network of genes controlling development and cellular maintenance in *Nothobranchius* species.

As a first step towards a comparative genomic approach, we have started to perform comparative gene analysis on different species of the genus *Nothobranchius* (of which we have established captive colonies) by sequencing *MTP*, *p66shc*, *IGFR1*, *SIRT1* and other aging-related genes. The use of several species for sequence comparison will allow us to use the ratio of conservative vs. radical mutations by likelihood methods as a test to detect association between sequence variations and lifespan in natural populations (Wong *et al*., 2004; Massingham & Goldman, 2005). This analysis will reveal whether the above-mentioned genes, which show effects on lifespan when targeted by spontaneous or artificially induced mutations, are also targets of molecular evolution and affect lifespan in natural populations.

In the future, progress in genome analysis will possibly allow the use of genome-wide comparisons between different species, which may identify new genes influencing lifespan in vertebrates.

This approach is complementary to the saturation mutagenesis suggested by Herrera & Jagadeeswaran (2004), and convergence of the two methods will eventually provide new insights into the genes influencing aging in vertebrates.

Aging-related genes in *Nothobranchius*

Some genes and molecular pathways that control aging are highly conserved throughout species, e.g. the IGF signalling pathway and the *Sir2* pathway. However, some genes appear to modulate aging specifically in mammals. The signalling adapter *p66shc* is a nuclear-encoded protein that localizes to mitochondria (Orsini *et al*., 2004) and has been shown to regulate the production of reactive oxygen species (Nemoto & Finkel, 2002) and to modulate lifespan in mice (Migliaccio *et al*., 1999). The microsomal triglyceride transfer protein (*MTP*) was implicated in the control of human aging by linkage analysis of centenarian families (Geesaman *et al*., 2003).

When dealing with non-model organisms, a frequently raised issue is that of paucity of genetic information about the 'novel' species. However, Herrera & Jagadeeswaran (2004) have already shown that they could easily isolate (by PCR amplification) *N. rachovii* homologues of defined zebrafish ESTs that also have similarity matches in the Fugu genome. Taking advantage of the available genomic data for three teleost species (zebrafish and the two pufferfishes *Takifugu rubripes* and *Tetraodon nigroviridis*) (see, for example, http://www.ensembl.org/), we have performed homology cloning of conserved aging-related genes in *N. furzeri*. We could easily amplify from cDNA extracted from whole fishes partial sequences of vertebrate-specific genes coding for *p66shc* (17.2% of the human sequences) and *MTP* (11.2% of the human sequence). Given the high conservation at the sequence level, we could also identify the homologues of *SIRT1* (13.4% of the human sequence) and *IGF1R* (7.3% of the human sequence). Our novel results are illustrated in Fig. 6, where we report a region of the amino acidic sequences of these genes we cloned in *Nothobranchius* and, notably, we illustrate their very high similarity to human homologues.

IGF-1 R

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              .. ::**..*:*************:*. ::*.******.**** : :*************:********************:**:***************
   Nf/41-140 FINKKRNNNRLGNGVLYASVNPEYISAAEMYTPDEWEVAREKITMHKELGQGSFGMVYEGIAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKE
Tr/1004-1103 VLNKKRNSDRLGNGVLYASVNPEYFSAAEMYVPDEWEVAREKICLSRELGQGSFGMVYEGLAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKE
 Dr/955-1054 IVTKKRNSDRIGNGVLYASVNPEYFSPFEMYVPDEWEVAREKITMCRELGQGSFGMVYEGIAKGVVKDEPETRVAIKTVNESASVRERIEFLNEASVMKE
 Hs/957-1056 VFHRKRNNSRLGNGVLYASVNPEYFSAADVYVPDEWEVAREKITMSRELGQGSFGMVYEGVAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKE
  Pp/654-753 VFHRKRNNSRLGNGVLYASVNPEYFSAADVYVPDEWEVAREKITMSRELGQGSFGMVYEGVAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKE
 Mm/958-1057 VFHRKRNNSRLGNGVLYASVNPEYFSAADVYVPDEWEVAREKITMNRELGQGSFGMVYEGVAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKE
 Rn/958-1057 VFHRKRNNSRLGNGVLYASVNPEYFSAADVYVPDEWEVAREKITMNRELGQGSFGMVYEGVAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKE
Cf/1454-1553 VFHRRRNNSRLGNGVLYASVNPEYFSAADVYVPDEWEVAREKITMSRELGQGSFGMVYEGVAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKE
 Gg/958-1057 VFNKKRNSDRLGNGVLYASVNPEYFSASDVYVPDEWEVPREKITMCRELGQGSFGMVYEGIAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKE
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MTP

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              ***:**:: *****::*:: .    :.**.*********::* **:**.     : **. **.*******. ****** *** *:*:*****:***:**
   Nf/26-125 SHNYNRFAKVGSSSAFSGFMARSADLTSTYSLDILYSGSGIMRSSNMNIYGSSNGAMLHGLQVAIEAQGLESLIAATPDAGEEDLESFAGMSALLFDVQL
Tr/1851-1950 SHNYDRFSKIGSSSAFSGFMAQSADLTSTYSLDILYSGSGILRRSNMNIFGASNGAMLHGLQVAIEAQGLESLIAATPDEGEEGLESFAGMSALLFDIQL
  Dr/600-699 SHNYDRFSKTGSSSAYSGFMAETVDVTCTYNLDILYSGSGVLRRSNMNIYGQSNNALLHGLQVTIEAQGLESPIAATPDEGEEELESFAGMSALLFDVQL
  Hs/602-701 AHNYDRFSRSGSSSAYTGYIERSPRSASTYSLDILYSGSGILRRSNLNIFQYIGKAGLHGSQVVIEAQGLEALIAATPDEGEENLDSYAGMSAILFDVQL
  Pt/589-688 AHNYDRFSRSGSSSAYTGYIERSPRSASTYSLDILYSGSGILRRSNLNIFQYIGKAGLHGSQVVIEAQGLEALIAATPDEGEENLDSYAGMSAILFDVQL
  Mm/601-700 VHNYDRFSKSGSSSAYTGYVERSPRAASTYSLDILYSGSGILRRSNLNIFQYIKGTELHGSQVVIEAQGLEGLIAATPDEGEENLDSYAGMSAILFDVQL
  Rn/602-701 VHNYDRFSKSGSSSAYTGYVERSPHAASTYSLDILYSGSGILRRSNLNIFQYIGKAELHGSQVVIEAQGLEGLIAATPDEGEENLDSYAGMSAILFDVQL
  Cf/762-861 AHNYDRFSKSGSSSAYTGYITRAPHLASTYSLDILYSGSGILRRSNLNIFQYIGKSHLHSSQVVIEAQGLEGLIAATPDEGEENLDSYAGMSAILFDVQL
  Gg/602-701 AHNYERFSKPGSSSAYSGYITRGPDISSTYGLDILYSGSGILRRSNLNIHVFDRNAQLHAGQVVIEAQGLESIIAATPDEGEENLDSFAGMSAILFDVQL
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p66shc

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              ***:**:: *****::*:: .    :.**.*********::* **:**.     : **. **.*******. ****** *** *:*:*****:***:**
   Nf/26-125 SHNYNRFAKVGSSSAFSGFMARSADLTSTYSLDILYSGSGIMRSSNMNIYGSSNGAMLHGLQVAIEAQGLESLIAATPDAGEEDLESFAGMSALLFDVQL
Tr/1851-1950 SHNYDRFSKIGSSSAFSGFMAQSADLTSTYSLDILYSGSGILRRSNMNIFGASNGAMLHGLQVAIEAQGLESLIAATPDEGEEGLESFAGMSALLFDIQL
  Dr/600-699 SHNYDRFSKTGSSSAYSGFMAETVDVTCTYNLDILYSGSGVLRRSNMNIYGQSNNALLHGLQVTIEAQGLESPIAATPDEGEEELESFAGMSALLFDVQL
  Hs/602-701 AHNYDRFSRSGSSSAYTGYIERSPRSASTYSLDILYSGSGILRRSNLNIFQYIGKAGLHGSQVVIEAQGLEALIAATPDEGEENLDSYAGMSAILFDVQL
  Pt/589-688 AHNYDRFSRSGSSSAYTGYIERSPRSASTYSLDILYSGSGILRRSNLNIFQYIGKAGLHGSQVVIEAQGLEALIAATPDEGEENLDSYAGMSAILFDVQL
  Mm/601-700 VHNYDRFSKSGSSSAYTGYVERSPRAASTYSLDILYSGSGILRRSNLNIFQYIKGTELHGSQVVIEAQGLEGLIAATPDEGEENLDSYAGMSAILFDVQL
  Rn/602-701 VHNYDRFSKSGSSSAYTGYVERSPHAASTYSLDILYSGSGILRRSNLNIFQYIGKAELHGSQVVIEAQGLEGLIAATPDEGEENLDSYAGMSAILFDVQL
  Cf/762-861 AHNYDRFSKSGSSSAYTGYITRAPHLASTYSLDILYSGSGILRRSNLNIFQYIGKSHLHSSQVVIEAQGLEGLIAATPDEGEENLDSYAGMSAILFDVQL
  Gg/602-701 AHNYERFSKPGSSSAYSGYITRGPDISSTYGLDILYSGSGILRRSNLNIHVFDRNAQLHAGQVVIEAQGLESIIAATPDEGEENLDSFAGMSAILFDVQL
```

SIRT1

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           ****:***:******************** **:**:: **: ******************:***:************:** *****.:* *:****.  *
  Nf/1-100 MFDIEYFRRDPRPFFKFAKEIYPGQFQPSPCHRFISMLDKQEKLLRNYTQNIDTLEQVAGVQRIIQCHGSFATASCLVCKQKVDCEAIREDVFNQVVPRC
 Tr/33-132 MFDIEYFRRDPRPFFKFAKEIYPGQFQPSLCHKFISMLDKQGKLLRNYTQNIDTLEQVAGVQRIIQCHGSFATASCLVCKHKVDCEVIREDIFNQVFILC
Tn/295-394 MFDIDYFRRDPRPFFKFAKEIYPGQFQPSLCHKFISMLDKQGKLLRNYTQNIDTLEQVAGVQRIIQCHGSFATASCLICKHKVDCEVIREDIFNQVVPHC
Hs/296-395 MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQRIIQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRC
Pt/315-414 MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQRIIQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRC
Mm/288-387 MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQRILQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRC
Rn/249-348 MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQRIIQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRC
Cf/371-470 MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQRIIQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRC
Gg/307-406 MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALMDKEGKLLRNYTQNIDTLEQVAGIQRIIQCHGSFATASCLICKYKVDCEVVRGDIFNQVVPRC
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Fig. 6 Amino acidic alignments of homologues of four aging-related genes cloned from *Nothobranchius furzeri* to their known (or computationally predicted) vertebrate counterparts. A, IGF1R; B, MTP; C, p66shc; D, SIRT1. Note very high similarity with human sequences. Species are identified by their initials: Nf, *Nothobranchius furzeri*; Tr, *Takifugu rubripes* (Fugu); Tn, *Tetraodon nigroviridis* (pufferfish); Dr, *Danio rerio* (zebrafish); Hs, *Homo sapiens*; Pp, *Pongo pygmaeus* (orang utan); Pt, *Pan troglodytes* (chimpanzee); Mm, *Mus musculus* (mouse); Rn, *Rattus norvegicus* (rat); Cf, *Canis familiaris* (dog); Gg, *Gallus gallus* (chicken); Xl, *Xenopus laevis* (African clawed frog). Amino acids that are identical, strongly similar or weakly similar among all represented species are indicated, respectively, with (*) (:) and (.). Numbers following each organism's name indicate the residue positions of the sequence fragment in the protein sequence. Note that only a part of each *Nothobranchius* cloned sequence is represented in the alignments, and that these sequences themselves constitute a part of the gene (not its full-length coding sequence). Alignments were performed with Clustal (Thompson *et al.*, 1997) and edited with Jalview (Clamp *et al.*, 2004) to trim sequence ends.

Genes shown to control aging provide important targets for the development of pharmaceuticals to prevent age-related pathologies in humans. As we show in Fig. 6, homologues of human aging-related genes are indeed present in *N. furzeri* and display considerable homology with their human counterparts. Therefore, we suggest that *N. furzeri* could be used to test the effects of pharmaceuticals aimed at vertebrate-specific genes, with all the experimental advantages provided by a short-living animal model.

Conclusions

The genus *Nothobranchius* consists of closely related fish of monophyletic origin all adapted to a seasonal life and that show short lifespan in captivity. There is evidence for low levels of genetic exchange between populations and large differences in lifespan which correlate with differences in the length of the wet season in their natural habitats. These fish are not difficult to maintain in captivity, nor are they difficult to obtain. They grow rapidly, are easy to reproduce and are highly productive. Development of the eggs can be modulated and embryos can be stored economically.

The overt aging phenotype is well characterized and reproducible across species. Ongoing research is revealing that many vertebrate aging biomarkers are expressed during their short lifespan. Furthermore, the survival pattern is reproducible over many generations. The phenomena of rapid growth, high initial reproductive capacity and low somatic maintenance are suggestive of a lifespan shaped by extrinsic mortality probability and are consistent with the disposable soma theory of aging.

The combination of relatively short lifespan and ease of captive maintenance render these fish excellent candidates for gerontological research across species and comparative genomics for the study of aging and development. A comparative study of *Nothobranchius* aging may yield insight into the evolution of the entire genome in response to differences in extrinsic mortality rates surpassing what the invertebrates *Caenorhabditis elegans* and *Drosophila* have yet offered.

Finally, the species *N. furzeri*, with its exceptionally short lifespan, offers the possibility to perform experiments such as large-scale pharmacological investigations or on experimental evolution, subjects thus far unthinkable in a vertebrate.

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