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REVIEW

Multifaceted Nothobranchius

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Abstract—Annual killifish of the genus *Nothobranchius* are seeing a rapid increase in scientific interest over the years. A variety of aspects surrounding the egg-laying Cyprinodontiformes is being extensively studied, including their aging. Inhabiting drying water bodies of Africa rarely allows survival through more than one rainy season for the *Nothobranchius* populations. Therefore, there is no lifespan-related bias in natural selection, which has ultimately led to the decreased efficiency of DNA repair system. Aging of the *Nothobranchius* species is studied both under normal conditions and under the influence of potential geroprotectors, as well as genetic modifications. Most biogerontological studies are conducted using the species *Nothobranchius furzeri* (GRZ isolate), which has a lifespan of 3 to 7 months. However, the list of model species of *Nothobranchius* is considerably wider, and the range of advanced research areas with their participation extends far beyond gerontology. This review summarizes the most interesting and promising topics developing in the studies of the fish of *Nothobranchius* genus. Both classical studies related to lifespan control and rather new ones are discussed, including mechanisms of diapause, challenges of systematics and phylogeny, evolution of sex determination mechanisms, changes in chromosome count, occurrence of multiple repeated DNA sequences in the genome, cognitive and behavioral features and social stratification, as well as methodological difficulties in working with *Nothobranchius*.

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INTRODUCTION

Modern gerontology, both fundamental and applied, is inconceivable without the studies carried out with the fish of *Nothobranchius* genus [1-5]. One of the representatives of this genus with the shortest lifespan, *N. furzeri* Jubb, 1971 (GRZ isolate) manages to get old and die naturally within only 3-7 months (not longer than 12 months for the species as a whole), which opens up huge possibilities in investigation of genetic and biochemical basis of aging, as well as in development of methodology for testing potential geroprotectors and analysis of their mechanism of action in vertebrates [6-8]. Previously such studies were primarily carried out using such models as *Caenorabditis elegans* and *Drosophila melanogaster* [9-13], but, obviously, the search for geroprotective interventions for humans could be more productive if the used model organisms are evolutionary closer to humans. Use of evolutionary distant models increases risks of rejecting interventions that could be potentially beneficial for humans. In addition, vertebrate testing is required as a final stage of investigation after conducting experiments with invertebrates. Hence, use of animals of the *Nothobranchius* genus allows to shorten time necessary for the development of medicinal drugs. The most popular model animals among vertebrates have a long lifespan: rodents live 2-3 years, and *Danio rerio* – up to 4-5 years. That is why Nothobranchius were such a "godsent" find for gerontologists [5, 14],

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moreover, the most important principle of bioethics with regard to selection of an animal model states that the study should be carried out with an organism at the lowest possible level of evolutionary development.

Testing of gene expression in different tissues, analysis of gut microbiome, as well as evaluation of cognitive and behavioral determinants during investigation of geroprotectors allows conducting comprehensive study of the effects of different interventions on an organism [15, 16] and continuing work for the goal of extending the active phase of life, i.e., ensuring active longevity [17, 18]. Considering demographic transition taking place in the Russian Federation and in many European countries, this research topic becomes extremely relevant.

The advantages of using Nothobranchius are not limited to investigation of mechanisms of aging. There are multiple unique features characteristic of the representatives of this genus, which attract attention of researchers. In particular, fine regulation of the processes of transition to the stage of embryonic diapause and its cessation, which is required for adjustment of the duration of the egg period according to weather conditions suitable for hatching (rainy season) [19-21]. Investigation of the ability of most complete arrest of metabolism is extremely important for the development of approaches for space exploration by humans.

The most promising directions of investigation of Nothobranchius are considered in this review that are being explored by the researchers worldwide – from sexual behavior and social hierarchy to structural features and functioning of the genome.

PROBLEMS OF SYSTEMATICS AND EVOLUTION OF THE *Nothobranchius* GENUS

Representatives of the *Nothobranchius* Peters genus 1868 include stenotopic spawning bony fish inhabiting drying freshwater reservoirs in Africa that belong to the Nothobranchiidae family, order Cyprinodontiformes. The *Nothobranchius* genus is rather large: several tens of valid species have been described, and each year new species are added to the list. On the other hand, habitat of the *Nothobranchius* genus is not very large, and conditions of existence are very limited by the environmental factors, although ecological niches occupied by different species are practically the same.

Researchers are interested in the causes and mechanisms of formation of such wide species diversity. This is directly related to the issue of identification of features distinguishing different species in the genus *Nothobranchius*.

What are the causes and mechanisms leading to emergence of such a large number of species in the *Nothobranchius* genus? A hypothesis has been suggested on this issue. Considering that the fish of *Nothobranchius* genus inhabit the seasonally drying water reservoirs in Central and Eastern Africa, their habitat is limited to small swamps with limited water movements that include flood-lands at the river sources, seasonally flooded plains, marshes, and streams of slowly moving, seasonally drying creeks and brooks. The area of Nothobranchius distribution is also limited to the regions with certain soil composition, because a sufficient level of soil moisture is required for long-term preservation of eggs. Nothobranchius were described in seven African regions: Nilo-Sudan, Victoria, Tanganyika, Malawi, East Coast, Zambezi, and Congo [22]. Representatives of the *Nothobranchius* genus are typical examples of allopatric speciation. It occurs under the effect of changes in topography of the land surface, and provides very limited habitats for each species, while the number of species is large. Distribution of this genus is mostly associated with the East African Rift System, and geobiological analysis confirms tectonic hypothesis of evolution of Nothobranchius.

Speciation is primarily associated with accumulation of changes in the genome. Large number of species within one genus implies that high rate of mutation accumulation is characteristic to the taxon as a whole. It could be suggested that such predisposition in Nothobranchius is associated with accelerated life cycle, in other words – with early aging, which, in turn, is associated with accumulation of genetic errors in each cell of the organism [22]. It could be hypothesized, as a result, that the system of DNA repair in Nothobranchius is not very effective because the efficient system capable of long-term life-long correction of errors in DNA is not required by this short-lived organism. This trait is not a subject of natural selection. Combination of high mutation rate with genetic drift over the short life cycle depending of wet season ensured high rate of species formation [23]. The cited features together with minimal differences in ecological niches indicate non-adaptive process of speciation in the genus *Nothobranchius* [24].

What taxonomic features are most adequate for distinguishing Nothobranchius species? Systematics of Nothobranchius is complicated by several issues. First of all, the species criteria are not totally clear, that is why the question arises often whether the new isolate is representative of already known species, or it should be recognized as a new species [25]. This problem is quite acute because almost every scientific expedition returns back with previously not described species of Nothobranchius. One of the important distinguishing signs is specific pattern of the male coloration [26]. The situation is complicated, on the one hand, by the fact of availability of various color morphs in the same species (such as in *N. furzeri*, *N. korthause*, *N. hassoni*), and, on the other hand, by the existence of twin isolates that are very similar phenotypically, but have different number of chromosomes.

The existing practice of naming new species of Nothobranchius is quite interesting. Very often a new

species is named after the name of the specialist, who obtained this isolate in nature and brought it from expedition. In the process, description of the species is carried out by different personnel often not including the discoverer as a co-author [27, 28]. There are very touching cases, when the researchers name new species after the name of their close relatives; for example, the species *N. ditte* was described by the well-known ichthyologist Béla Nagy, who named this species in honor of his wife Edith [29].

One of the important criteria in systematics of species is the ability of free inbreeding of organisms producing fertile offsprings that preserve all features of the species for generation. But conducting this type of tests with Nothobranchius under laboratory conditions is limited by the usual absence of a representative collection of the species. Sometimes there are situations in nature when several species of Nothobranchius inhabit the same territory. These species are termed sympatric in the literature. However, firstly, usually there is no sufficient time during expedition for the detailed analysis of the species composition in the water reservoir, and, secondly, identification of the potential hybrids is not always possible due to the absence of a developed system of markers.

There were hopes that molecular systematics could help, but the researchers encountered problems in this area also. It was found out that very limited habitats do not provide any possibility to form a notion on intraspecies variability, i.e., molecular methods do not provide an exact answer to whether the similar isolates belong to the same species or to different ones. However, it can be stated with confidence that the genus *Nothobranchius* is a unique object for investigation of microevolution, and that there will be numerous discoveries in this area in the future [22].

In conclusion, it should be mentioned that investigation of African flora and fauna is complicated by fast urbanization of the territories that results in the changes of environmental conditions and, hence, decrease in biodiversity. That is why in the nearest future our planet could lose many Nothobranchius species, both those already described and those that are not discovered yet.

KARYOTYPIC DIVERSITY, FEATURES OF GENOME ORGANIZATION, AND EVOLUTION OF SEX DETERMINATION

For a long time in pre-genomic era, karyology was an important tool of systematics, which allowed to identify additional features, if morphology did not provide enough information. Karyotyping also helped to explain causes of the hybrid sterility, detect polyploidization, determine sex at early age prior to emergence of phenotypic characteristics. However, the experts in karyology of Nothobranchius were not able up until now to agree even on some basic issues. The thing is that karyotype of different representatives of the *Nothobranchius* genus is characterized with unusually high variability [30]. Number of chromosomes varies from 16 to 50. The causes and mechanisms leading to existence of such variability, as well as direction of the evolutionary process are of great interest to the scientists. Currently the issue became clearer, although there are only few studies investigating karyotype due to popularity of genome sequencing, replacing karyotyping.

The issues of determination of sex in the representatives of Nothobranchius also attract a lot of attention. Many aquarium fish breeders noticed that the 1 : 1 male/female ratio almost never observed in the fry of the *Nothobranchius* genus and during their further growth. In particular, there is around five males per one female in the *N. guentheri*. It is also generally accepted that sex determination in the *Nothobranchius* genus occurs under the effect of environmental factors and due to genetic characteristics.

Conducting omics studies is nowadays the most advanced and popular strategy including first of all genome and transcriptome sequencing [18]. Information on DNA sequence in the genome of the species opens wide possibilities of molecular and genetic studies, as well as allows determining position of the species in the phylogenetic tree. However, assembly of the Nothobranchius genome is highly complicated due to the presence of a huge number of repeats, origin and role of which is a subject of numerous discussions.

How the Nothobranchius karyotype evolved, and how many chromosomes was in the ancestor species? Considering the causes and mechanisms of karyotype evolution in Nothobranchius, it should be noted that chromosomal rearrangements are natural and common events in the cases of high rate of speciation. The most frequent changes are associated with the shifts of centromeres on chromosomes and inversions, which could facilitate local adaptation due to suppression of recombination and, hence, accumulation of linked adaptive genes. It is important to note that such evens often result in genetic incompatibility, decreasing fertility of the hybrids, facilitating reproductive isolation and species formation [31-34].

Karyotypes of more than 65 species have been described [35]. All known Nothobranchius are diploid (2n), however, number of chromosomes in different species varies from 16 to 50 (most frequently 36, next by frequency – 38) [36]. This feature makes Nothobranchius a very attractive model for the researchers working in the area of evolution and ecology.

Considering the extremely large range of chromosome counts – in different species, it could be suggested firstly that at some stage of evolution polyploidization occurred, which was followed by divergence of duplicated chromosomes according to the structure and functions. In this case the species with the lowest number of chromosomes would be evolutionary most ancient. The second idea was considering the multi-chromosome species (with diploid set of more than 38 chromosomes

including *N. ditte*, *N. malaissei*, *N. brieni*) as ancient forms. However, the existing phylogenetic trees, as well as detailed investigation of karyotypes of different species indicate that most likely the oldest was the species with 38 chromosomes.

Karyotype changes in the subgenus *Nothobranchius* [37] (2n = 38) occurred mainly via pericentric inversions. Four species – *N. furzeri*, *N. kadleci*, *N. orthonotus*, and *N. kuhntae* – differed from each other by the ratio of uniarmed and biarmed chromosomes [35]. Reduction of chromosome number through their fusion is typical for the species with the number of chromosomes below 38. Moreover, biarmed chromosomes were predominant in the karyotypes of the species with the lowest number of chromosomes (*N. rachovii* – 16, *N. krysanovi* – 18).

Abundance of not only intra-chromosomal but also inter-chromosomal rearrangements is typical for the *Nothobranchius* genus. Two major trends are recognized in the evolution of chromosomes: chromosome fusion (or, less often, splitting into two chromosomes) and pericentric inversions [35].

It could be stated in conclusion that according to the modern notions the chromosome set with 2n = 38 is primary in evolution. Next the processes of increase and decrease of the number of chromosomes occurred in parallel in several groups of fish. All cases with the increased number of chromosomes originated independently of each other. The mechanisms of increase of the number of chromosomes are associated not with polyploidization, but with splitting of the existing chromosomes into smaller ones. The processes of decrease of the number of chromosomes are evolutionary associated with their fusion.

How sex is determined in different Nothobranchius species? Among the described karyotypes of the representatives of *Nothobranchius* genus, five species have morphologically defined sex chromosomes including *N. guentheri* [38], *N. brieni* [36], *N. lourensi*, *N. janpapi*, and *N. ditte* [35]. However, even the chromosomal mechanisms of sex determination differ in all investigated species. Let us consider, for example, the genotype of *Nothobranchius brieni* [36]. The number of chromosomes in *N. brieni* is 2n = 49 for males and 2n = 50 for females. The female karyotype consisted of 25 pairs of acrocentric chromosomes gradually decreasing in size. The male karyotype consisted of 23 pairs of acrocentric chromosomes, one biarmed chromosome pair and two unpaired acrocentric chromosomes. In the first meiotic chromosomes during spermatogenesis 23 bivalents were observed, and one trivalent at diakinesis. Hence, the *N. brieni* species possesses a multi-sex chromosome system of the type $X_1X_2Y/X_1X_1X_2X_2$. One biarmed neo-Y-chromosome most likely emerged as a result of Robertsonian fusion of the Y-chromosome and autosome, as described for other fish species [39] In *N. brieni* and *Nothobranchius* sp. "Kasenga" Y-chromosome is large and metacentric, and X1- and X2 chromosomes are acrocentric and of different size [39]. In four not-closely related subgenera of *Zononothobranchius* (*N. brieni*) and *Adiniops* (*N. guentheri*, *N. lourensi*, and *N. janpapi*) [40] multiple sex chromosomes supposedly originated independent on each other [35].

In some species it was impossible to detect morphologically different sex chromosomes, but, on the other hand, SDR-loci have been identified that contain sex-specific DNA sequences. Let us consider *N. furzeri*, as an example, which has XY sex determination system with the sex chromosomes practically undistinguishable from the morphological point of view. Males are heterogametic carrying a unique variant of the *gdf6* gene, which is a representative of the TGF-β growth factor family initiating development of the male-type [41]. Y-variant of the *gdf6* differs from the X-variant by 15-amino acid substitutions and 3 deletions. It is important to note that the substitutions occurred in the highly conserved region common in vertebrates, and this indicates strong positive selection towards accumulation of mutations in this gene.

Immediately after hatching both alleles of *gdf6* are expressed at approximately the same degree, while 3 days after expression in males is significantly higher than in females. In mature fish there is sex-dependent expression of the *gdf6* gene: while in the ovaries of female fish expression is very low, in the testes of the male fish it is detected, which could be associated with one more deletion occurring at 3'-UTR cutting off the site of binding of mir-430 – important regulator of gene expression in the sex line of fish [41]. Details of the mechanism of the action of *gdf6* and prevalence of the sex determination system associated with it in other species of Nothobranchius requires further investigation.

Availability of such variable systems of sex chromosomes makes the *Nothobranchius* genus a very promising model for investigation of the pathways of establishing sex determination systems.

The question remains, why the male/female ratio in the *N. guentheri* is far from the expected 1 : 1? Growth rate and, consequently, the body size in males is greater that the same parameters in females, that is why in the cases of combined breeding of fry females are outcompeted and either become belly-sliders, or are eaten by the larger beings. When the fry were sorted in our laboratory into different containers according to their size the ratio of sexes immediately became close to the standard one 1 : 1.

What are unique features of the Nothobranchius genome? Development of the methods of new generation sequencing allowed not only sequencing the genomes of several species of the *Nothobranchius* genus, but also start working in the area of functional genomics. Analysis of transcriptomes, investigation of non-coding RNAs, methylome, and gut microbiome composition are used more often nowadays [16]. Examination of gut microbiome composition could be carried out either using cultur-

al methods or with the help of new generation sequencing [16, 42], which allows analyzing metabolic potential of microorganisms and their effect of the host metabolism.

The *N. furzeri* haploid genome consists of 19 chromosomes (2n = 38). Genome size is approximately ~1.5 billion nucleotide base pairs [41] with more than 22,000 of protein coding genes annotated [43]. Whole genome sequencing significantly simplified application of molecular biology methods and approaches. High level of inbreeding was demonstrated for the most short-lived and most sought for isolate *N. furzeri* GRZ based on the genome analysis. It was shown that the *N. furzeri* genome contains a large fraction of repeats reaching 45%, which is considered very high for bony fish [41, 43]. This feature complicates assembly of the Nothobranchius genome, although evolutionary origin of this feature and its role in metabolism of these fish attract attention. According to our hypothesis origin of a large number of repeats is associated with enhanced activity of the genome elements capable of transposition. In particular, we observed the increased activity of transposons during comparison of transcriptomes of *N. guentheri* males of different hierarchical status. Transposons contain in their composition regulatory sequences that exert *cis*- and *trans*-effects on expression of specific protein coding genes. Hence, they are capable of changing mechanisms of morphogenesis thus playing a role in adaptation of an organism to environmental factors.

Tandem repeats, mobile elements (transposons and retrotransposons), pseudogenes, and segment duplication are present in the *N. furzeri* genome. Among those tandem repeats comprise record 20% of the genome, which makes *N. furzeri* outstanding in comparison with other fish. Among the tandem repeats two GC-rich mini-satellites localized in the pericentromeric region with length 77 and 49 nucleotides are highly represented. The 348-nucleotide satellite sequence with low G+C content also is localized close to centromeres. The mobile elements comprise ~25% of the genome, and this is an important feature of the *N. furzeri* genome [41].

Both nuclear and mitochondrial genomes of Nothobranchius are rather large in comparison with the genomes of related fish species, which are not short-lived. Increase of the size of nuclear genome to a great extent is due to the increased number of genome elements capable of transposition caused most likely by the genetic drift. Mitochondrial genome of *N. furzeri* is also larger (>19.5 kb) than in other vertebrates (~16 kb), and is characterized by the larger total length of non-coding regions [43].

Karyotype variability within the *Nothobranchius* genus is very large in comparison with other genera of bony fish [44, 45], which implies that this originated in the course of evolution as a result of frequent large-scale genome rearrangements, which, in turn, is in agreement with the explosive speciation within the *Nothobranchius* genus [43].

ADAPTIVE POTENTIAL OF NOTHOBRANCHIUS

Nothobranchius in their natural habitats are practically always surrounded by hazardous environmental factors. They were able to develop a number of adaptation mechanisms in the course of evolution that help them to survive and actively evolve. Detailed elucidation of these mechanisms is of crucial interest to numerous researchers, because this is important not only from the fundamental point of view, but also has practical significance.

What are main directions of adaptation that are characteristic only to Nothobranchius? Habitats of Nothobranchius in the seasonally dry/wet regions of African savanna are extremely variable. Many factors change very fast and in very broad range (such as daily temperature fluctuations from 14 to 37°C) [1]. In order to survive under such conditions, animals need to adapt. It is likely that Nothobranchius as members of ephemeral fauna had a certain level of pre-adaptation to environmental conditions, because their genome contains rather large number of mobile elements capable of transposition, which, in turn, mediates insertional mutagenesis. This process forms the basis of genome changes in the course of natural selection [46]. The ability of transposons to move into the specific genome sites in the course of evolution, regulate gene expression and interact with transcription factors in combination with the ability to respond to ecological stressors, forms the basis for rapid variability and speciation due to modulation of regulation of ontogenesis. In this way Nothobranchius adapted to the constantly changing conditions due to, at least in part, presence of the increased number of transposons in their genome.

In addition, representatives of the *Nothobranchius* genus have a unique set of reproductive adaptations, one of which is existence of diapause, i.e., ability of embryos to slow down their metabolism significantly under certain conditions. One of the main advantages is the possibility to regulate total time of the embryo staying within the protective coat of the egg, which is crucial in the case of fluctuations of weather conditions from one year to another [44, 45].

In addition to regulation of the length of time under protection of the egg coat, another important adaptation mechanism includes survival of embryos during drought due to formation of thick coat of the egg, structure and composition of which has been formed in the course of evolution under conditions being surrounded by smectic clay.

Fish egg are able to withstand desiccation and develop in such soil for a long time. And exactly these hydromorphic properties of the cracking clay substrates play a key role in uneven distribution of these species in tropical landscapes. The fish of *Nothobranchius* genus lay eggs into a soft silt with typical properties vertisol. It is critically important that the minerals in composition of smectite (most often montmorillonite) have the ability absorb water molecules in between crystalline layers. During droughts water evaporates resulting in formation of deep

cracks in the soil. Hence, during the dry period fish eggs deep inside the cracking clays, where optimal moisture level is maintained, could enter the state of diapause and remain viable until the next wet period [26, 44]. In the course of evolution diapause became not only a vital adaptation required to survive draught but also became necessary for formation of a healthy juvenile.

Diapause phenomenon in vertebrates and mechanisms of its control. Diapause is mostly observed in invertebrates, that is why investigation of diapause using fish model is especially valuable form the point of view of practical application of the results. Major part of the studies has been carried out with the relatives of Nothobranchius from South America; egg incubation period is shorter for these fish; however, it is generally assumed that the mechanisms of development are very similar.

Three stages of diapause are recognized, which are considered as stages of one process, although duration of each stage could vary significantly. Such system suggests existence of various regulation mechanisms for each stage of diapause. Environmental conditions play an important role in regulation of these stages [47]. Molecular mechanism defining the possibility of diapause initiation have been investigated such as its dependence on the vitamin D signalling pathway [21]. It has been suggested that there is a special pathway that integrates all information on environmental conditions and transforms it into developmental programs associated with evolutionary transformation in animals. Hormones derived from 7-dehydrocholesterol are considered as signalling molecules together with associated nuclear receptors.

In nature diapause I occurs already in the rainy season during dispersion phase before gastrulation, at this stage entire pool of embryos develops synchronously. Embryos enter the phase II synchronously, which begins in the middle of somitogenesis, stage of formation of majority of organs. Diapause II is characterized by reduced protein synthesis, arrest of cell cycle, remodelling of energy metabolism, and all is controlled by the system of insulin-like growth factor. Mitochondria isolated from the embryos at the diapause II are not ready to generate ATP, but rather transport carbon and electrons along the Krebs cycle minimizing generation of proton-motive force [48]. Diapause III occurs at the final stage of development immediately before hatching [47]. Asynchrony occurred at the beginning and end of the dry season.

All three stages of diapause are required, but their duration could vary depending on the external stimuli. In *Austrofundulus limnaeus* the process of entering and exiting diapause is linked to the temperature of the environment, as well as to certain signals transferred from the maternal organism [20, 49]. In particular, probability of the embryo entering long diapause depends on the age of female and number of eggs it already produced [49]. We observed similar pattern during breeding of *N. guentheri* and *N. rachovii* under laboratory conditions.

It was found out that the process of embryonic development, cause of diapause, in particular, differs in the fish living in wild nature and those living under laboratory conditions. The main distinguishing sign is reduction of diapause duration. For example, diapause III is practically not observed in the *N. furzeri* under standard incubation conditions. Most likely it becomes very short. This could affect the results of scientific experiments, and, hence, requires further detailed investigation to elucidate differences.

COGNITIVE-BEHAVIORAL SPHERE AND PECULARITIES OF SOCIAL BEHAVIOR

Unlike the traditional model organism, *Danio rerio*, representatives of the *Nothobranchius* genus do not live in shoals and are carnivores. World Health Organization even considered using Nothobranchius as biological means to fight malaria in Africa, suggesting that they are able to destroy mosquito larvae [50]. It seems likely that it is carnivorism that facilitated development of cognitive abilities during evolution of Nothobranchius and mediated emergence of other behavioral patterns not typical to non-carnivore fish.

Is it possible to say that fish have their own personalities? There is a very interesting area of studies aiming at investigation of stable individual differences in behavior. The term “animal personality” has been introduced [51] and variations in behavior were confirmed for some fish species [52, 53].

It was proved experimentally that fish of the *N. furzeri* species exhibit individual variations of behavioral patterns. In order to prove this peculiarity, the tests were conducted estimating propensity to investigative behavior in fish, assessing its boldness and risk tolerance. Results of this study confirmed existence of differences in the sequential behavioral reactions, and all behavioral indicators associated with motor activity and risk proneness were found to be repeatable [54].

Individual behavioral differences between fish were also observed in our studies. To test an experimental preparation, fish of the *N. foershi* species were placed individually into 50-ml tubes for 1 h four times a day. After the time in a tube, fishes demonstrated two different behavioral patterns. One group rapidly swam away and hid near the farther end of aquarium, while another group swam to the front of aquarium and demonstrated signs of aggressive behavior.

How hierarchic social system is organized in Nothobranchius and how to take this into account during experiments? It is well-known that animals of the same species in majority of cases form hierarchic social structure when under conditions of limited resources [55]. In the process, dominant, subdominant, and low-rank individuals emerge [56].

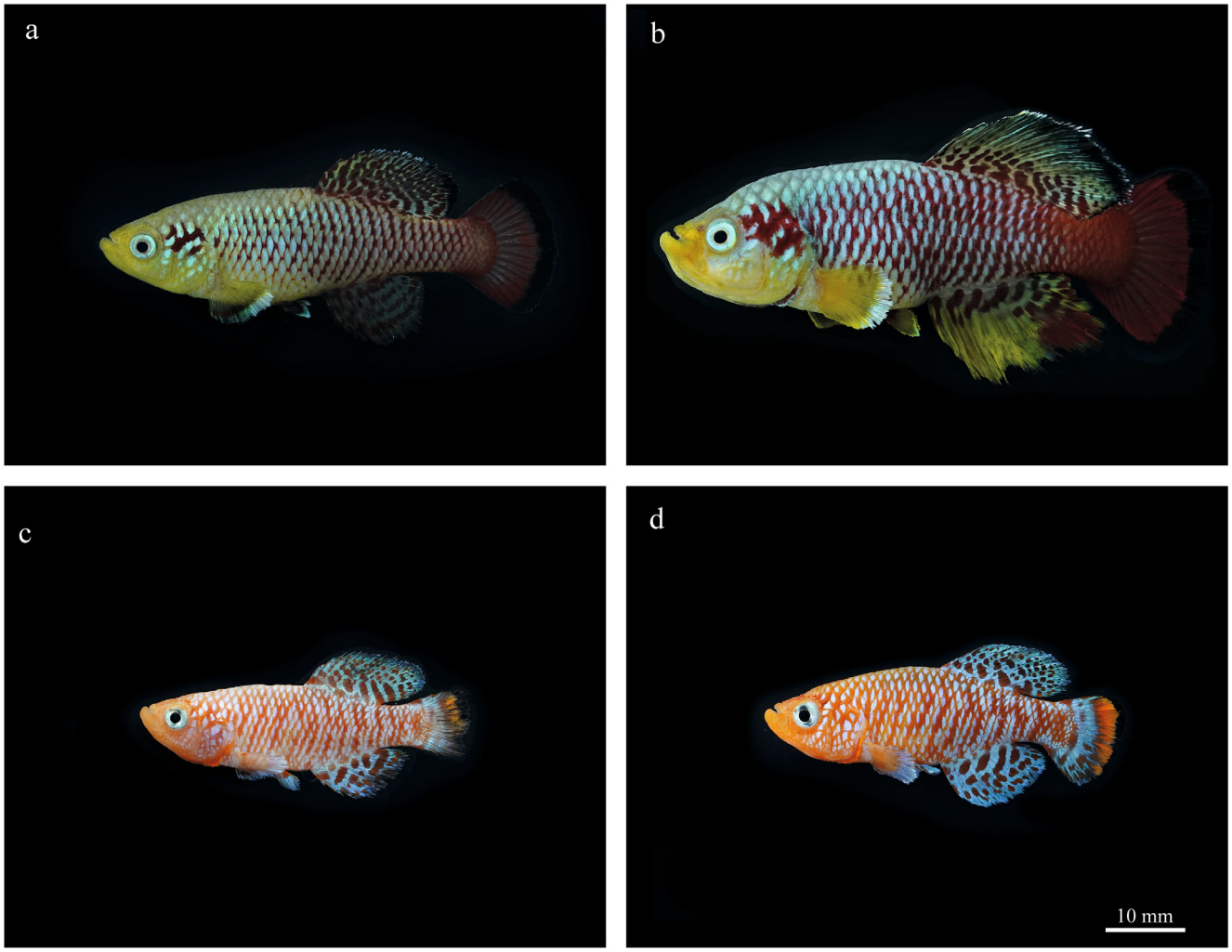

Fig. 1. Social stratification in the artificially formed groups of *Nothobranchius guentheri* (a and b) and *Nothobranchius rachovii* (c and d) males in the case of excessive feeding with *Chironomus* larvae. Level in the hierarchic ladder is reflected by the size of individual, peculiarities of coloration, as well as behavioral patterns. Dominant males are larger and have brighter coloration (b and d), while the low-rank individuals are smaller, have pale coloration and less pronounced coloration patterns of the body surface (a and c).

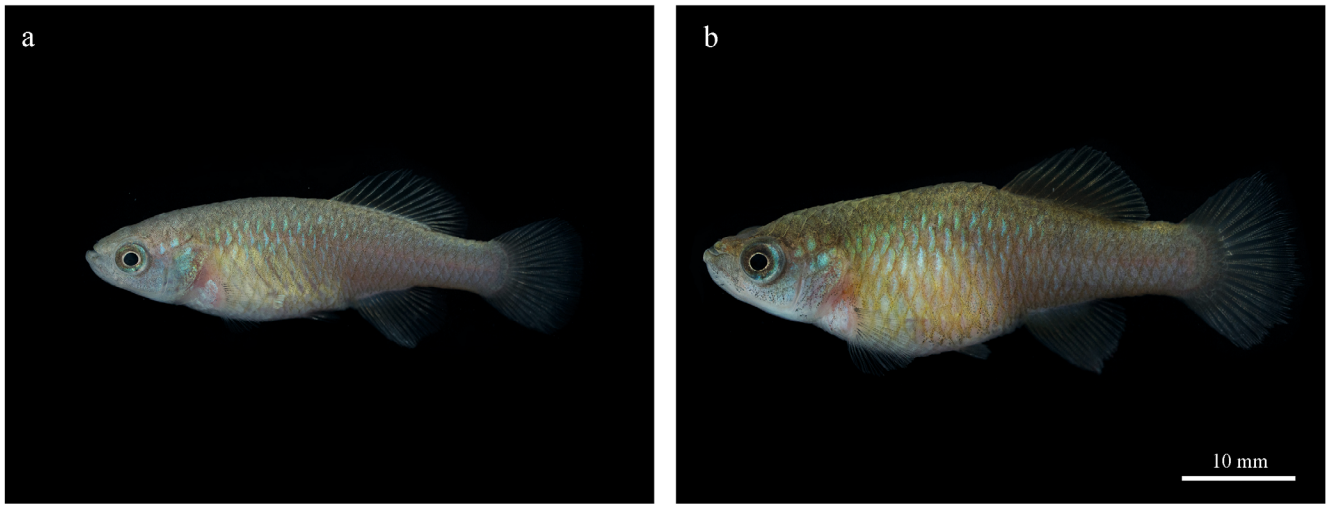

Fig. 2. Under aquaculture conditions among the normal *Nothobranchius guentheri* females of average size (a) masculinized females emerge (b). They could differ significantly in size, have darker coloration, and exhibit high level of aggression towards females and males of the same species.

In the initial stages of working with Nothobranchius (*N. guentheri*, *N. rachovii*, and *N. flammicomantis*) we observed that fish actively interacted with each other, when kept in groups under laboratory conditions. Type of interactions univocally indicated that it is impossible to conduct any long-term experiments even in the case, when the group of fish was formed already at early age, and new individuals were not added to the aquarium.

In the case of insufficient feeding (3-4 times a day to satiation with brine shrimp *Artemia salina*), Nothobranchius become more aggressive, interaction of individuals with the group often resulted in trauma and sometimes death. Interestingly enough that such peculiarities of social interactions were observed even in the most non-aggressive species, *N. rachovii*. In the case of excessive feeding (4-5 times a day with larvae of mosquitoes of the *Chironomidae* family), the fish exhibited aggressiveness aimed at killing opponent less often, but males demonstrated behavioral patters towards the individuals of the same sex typical of mating. Moreover, diversification in terms of morphological features and behavioral patterns could be observed (Fig. 1).

Breeding of *N. guentheri* females both in isolation and together with males also results in diversification of the individuals with regard to morphological parameters. While in the case of males, gradual transition between the largest and most brightly colored individuals is observed, there is another situation in the case of females. The most pronounced event is emergence of large and aggressive females with darker coloration, although they never acquire phenotype and functionality of males (Fig. 2).

The causes determining the role of each particular being in a social structure are not know at present. Hierarchical structure is common in fish [57], but in Nothobranchius it is still poorly understood; and it is not only of fundamental interest, it is also important for correct group selection for conducting various types of experiments. For example, it could be important in testing potential geroprotectors and performing corresponding behavioral tests. Representatives of the *Nothobranchius* genus could be used as a model for investigation of molecular and genetic mechanisms of various behavioral patterns.

In our experiments we found some molecular mechanisms that are potentially associated with the dif-

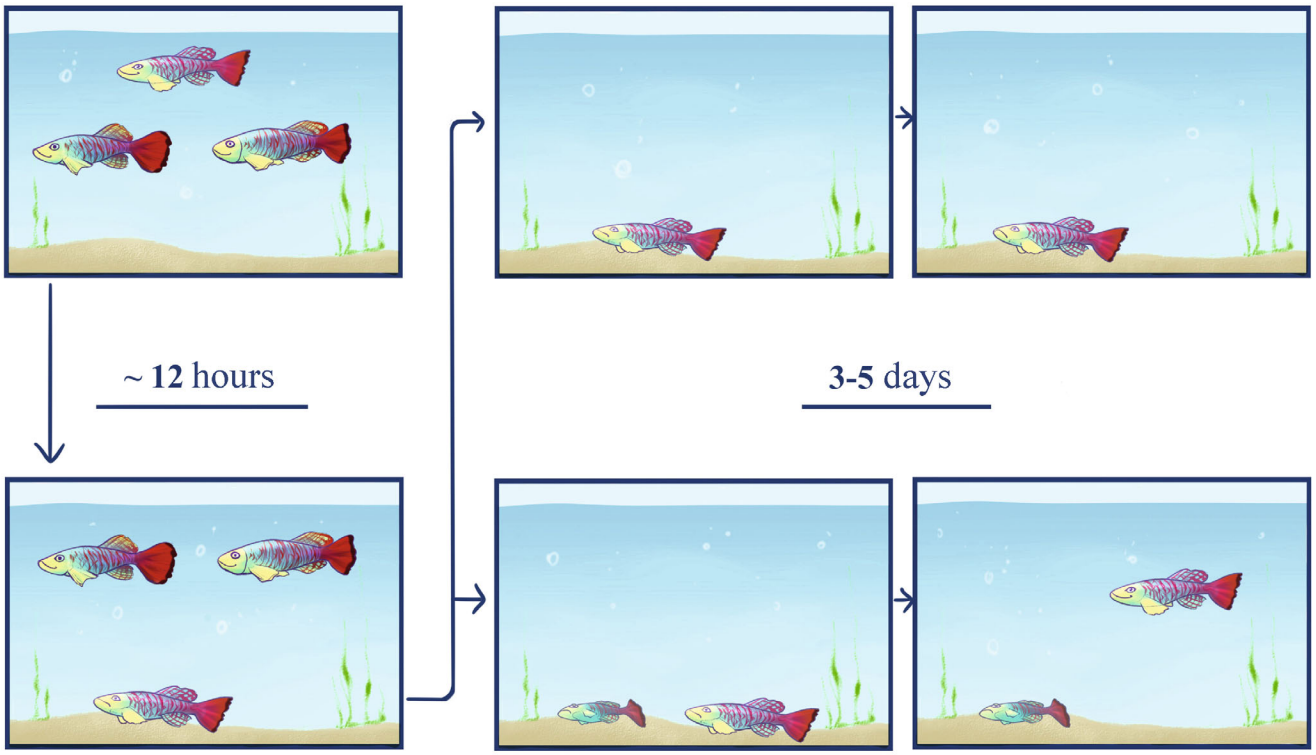

Fig. 3. Social stress in *N. guentheri* males. Potential effect of bullying on both participants of the interaction. If three male fishes spent one night in one aquarium with excess of food, there is high probability that in the morning one of them becomes a belly-slider. In the case when experiment starts in the daytime, the most probable result is death of one of the males due to aggressive behavior and attacks from other participants. After becoming a belly-slider the organism is seeking safe place in the aquarium, where it is not seen by the others. If the belly-slider in the morning is placed into a separate aquarium thus preventing effects of stress provided by the neighbours, there is a possibility that the fish will return to initial status within 3-5 days and stop being belly-slider. If a smaller belly-slider (male or female) is placed into aquarium with larger belly-slider, a new hierarchy is formed. The tested male organism occupies the dominant position and attack the smaller one. It was established as a result of the experiment that the presence of lower-rank male, which could be subjected to bullying, increase the probability of restoring the normal state of the control of body position in water.

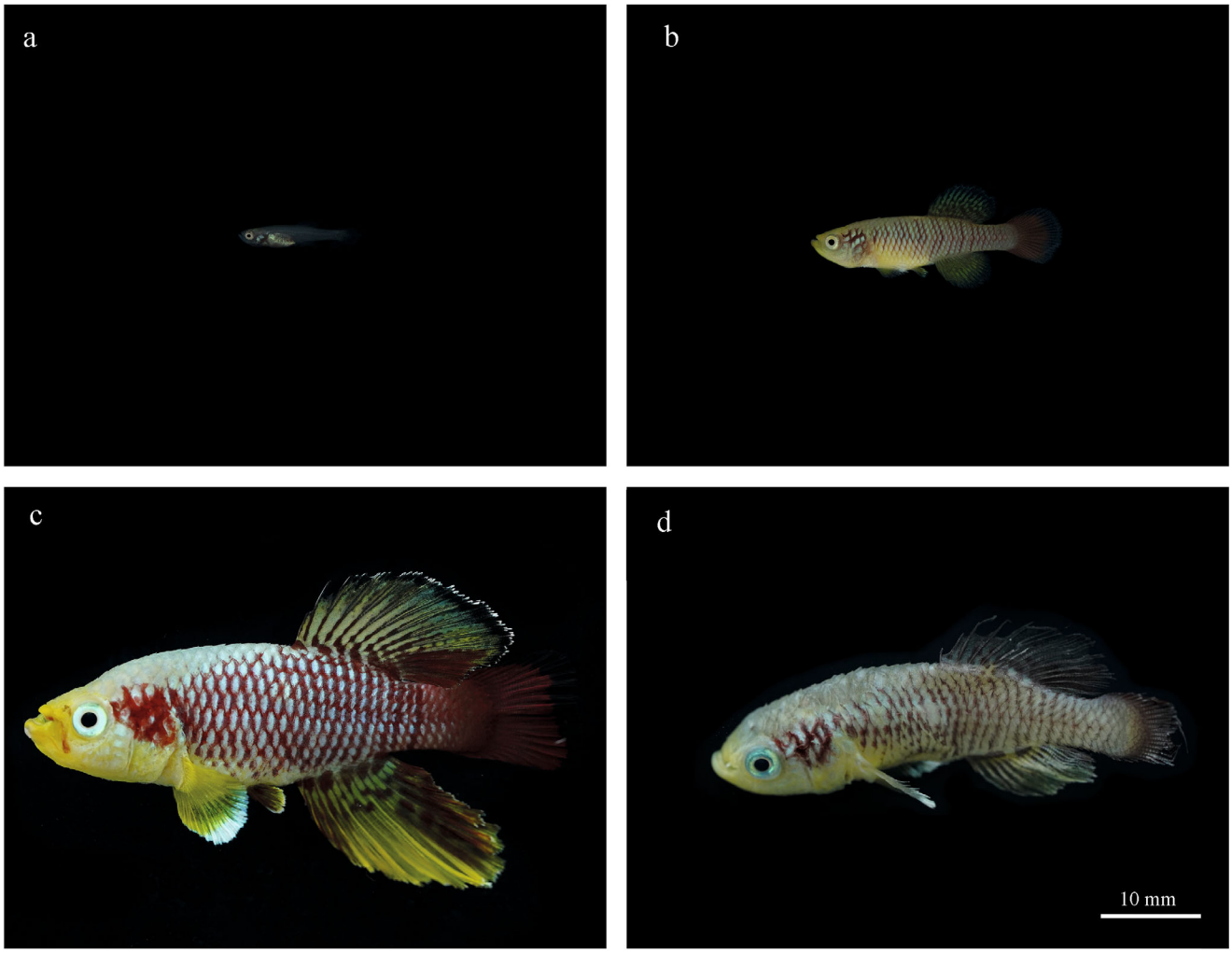

Fig. 4. *Nothobranchius guentheri* males of different age: a) 7 days; b) 2 weeks; c) 3 months; d) 18 months.

ferences in behavior of Nothobranchius and their hierarchic status. Analysis of the transcriptome of *N. guentheri* males of different social rank (dominant and low-rank individuums) was conducted. Significant increase of the level of expression of transposons was observed in the dominant males, which was predominantly associated with expression of protein encoding genes involved in the activity of nervous system cells. It is likely that the high social rank serves as a stress stimulus, which activates expression of transposons.

Taking into consideration the observed changes at the level of transcriptome dependent on the hierarchic status, it could be suggested that these changes could impact the results of testing of, for example, geroprotectors or during other investigations. The obtained results must be kept in mind during experiment planning and selection of reference groups.

How social stress could affect functioning of an organism and results of translation studies? High sensitivity to social stress is common in Nothobranchius. In our studies the most effective manifestation of this feature could be rapid loss of the ability to control body position in the water. This could be observed most often in males subjected to bullying by other males in the aquarium, and in females during formation of the spawning group, when one of them does not get sufficient attention of males. These individuals move predominantly along the bottom, and that is why they are called belly-sliders; social stress is not the only cause of such pathology [58, 59]. Significance of social stress, mechanism of its realization, and psychogenic potential of its effect on physiology of the organism is presented in Fig. 3.

The obtained results could explain, at least in part, the well-known problem associated with challenges in translation of the results of experiments with model animals to humans: model organisms are kept under practically ideal conditions during experiments, while humans are constantly under social stress during medicinal therapy and clinical trials. The stress is associated with both acknowledgment of the disease and social complications in communicating this fact, which are directly related to the disease, and with common routine situations, to

which humans are subjected in everyday life. We hypothesize that in order to obtain more relevant results of clinical trials it is necessary to supplement them with the factors that surround humans in real life. Susceptibility of Nothobranchius to social stress could provide us with this possibility.

AGING

The majority of animals exhibit similar aging patterns, although some exceptions exist. The differences are usually lie in the life expectancy and dynamics of manifestation of age-related symptoms/diseases, rate of aging.

It is impossible to investigate mechanisms of aging using only humans, especially when conducting testing of some potential medicines. For this purpose, model animals are required. Selection of models depends on the factors such as evolutionary closeness, similarity of aging processes with humans, and availability of characteristics of aging, which could be used as biomarkers of aging processes. Small mass of a model animal and minimal life expectancy also could be beneficial. Fulfilling the last two requirements would allow not only to obtain results faster, but also to significantly reduce cost of testing of investigated preparations. Before deciding whether Nothobranchius could be a valid model organism, it is important to confirm that the selected model is adequate for extrapolation of the results of investigation.

With a relatively short life cycle, the representatives of *Nothobranchius* genus go through aging stages typical for mammals including humans. This is manifested in several ways including gradual development of aging signs: Nothobranchius lose intensity and contrast of coloration, lose body mass and weight, accumulate mutations, demonstrate reduced locomotor activity. Synchrony of the calendar and biological age is disrupted more in Nothobranchius with age, similar to humans. So that individuums of the same calendar age could differ from each other in number and specific set of aging signs.

What aging signs should be considered during development of the methods for correction of aging in Nothobranchius? Being model organisms in gerontology, Nothobranchius have a number of morphological features, which could be used as markers of aging. Status of these markers and its change must be monitored in the course of experiment. Evaluation of aging markers allows monitoring dynamics of age-related pathologies and evaluate biological age during testing of various interventions. Visible external signs of aging also must be considered from the point of view of methodology, for example, during sampling for obtaining statistically significant results, especially if the testing is conducted with older fish, because the individuums in the group with time could become of very different biological age.

Males lose intensity of the body coloration; coloration pattern of the anal and dorsal fins becomes pale and lose contrast. Some researchers consider these processes in Nothobranchius as an analogy of the processes occurring in mammals, which lose hair and skin pigmentation with age [60]. A *N. guentheri* male at four stages of development (before coloration development, in the process of acquiring coloration, at maturation, and at old age) is presented in Fig. 4.

Integrity of fins is disrupted in aging fish, even under conditions when fish are kept individually, which prevents risk of trauma inflicted by other fish. Significant changes are observed in musculoskeletal system – spinal curvature is observed both in males and females. Hump is formed in some individuums, as well as muscle wasting and weight loss are observed. Disorders in visual system are also typical for aging fish. Rather often white cloudy spots are formed in the eye, especially in females, which could grow and completely cover cornea similar to cataract in humans (Fig. 5). Gradual irreversible decrease of pupil up to the size of a tiny dot more often observed in males.

With age Nothobranchius lose the ability to regenerate tissue; for example, 8-week-old *N. furzeri* (strain MZM-0703) are capable of almost complete regeneration of the tail fin within 4 weeks, while at the age of 54 weeks tail fin regeneration reaches only 46% of the initial size [61]. Similar to majority of animals, fish of the *Nothobranchius* genus gradually lose their reproductive functions, which starts reducing in *N. furzeri* by the age of 8-10 weeks [58].

Similar to humans the features of aging in Nothobranchius include development of neoplasia. Tumors investigated in different strains of *N. furzeri* had disorders in such proteins as Bcl-2, cytokeratin-8, carcinoembryonic antigen, and p53. Liver is the organ most susceptible to tumor development in Nothobranchius [62]. The probability of neoplasia increases with the fish aging [63], which is also observed in humans [64]. Interestingly enough, incidence of liver cancer is higher for the *N. furzeri* males than for females [62], which is also true for the incidence rates of liver cancer in humans [41, 65].

The cited symptoms of aging in Nothobranchius correspond to some age-related changes occurring in humans, mice, and other vertebrates [66]. Hence, researches have a set of biomarkers of aging in the representatives of the *Nothobranchius* genus, which allows investigation of the mechanisms of age-related pathologies in different organ systems, evaluate geroprotective effects of the tested interventions, and monitor age-associated changes using non-invasive techniques and produce results relevant to vertebrates within short period of time.

What molecular and genetic features in Nothobranchius are most promising for investigating aging? In 2015 the genome of *N. furzeri* was sequenced, assem-

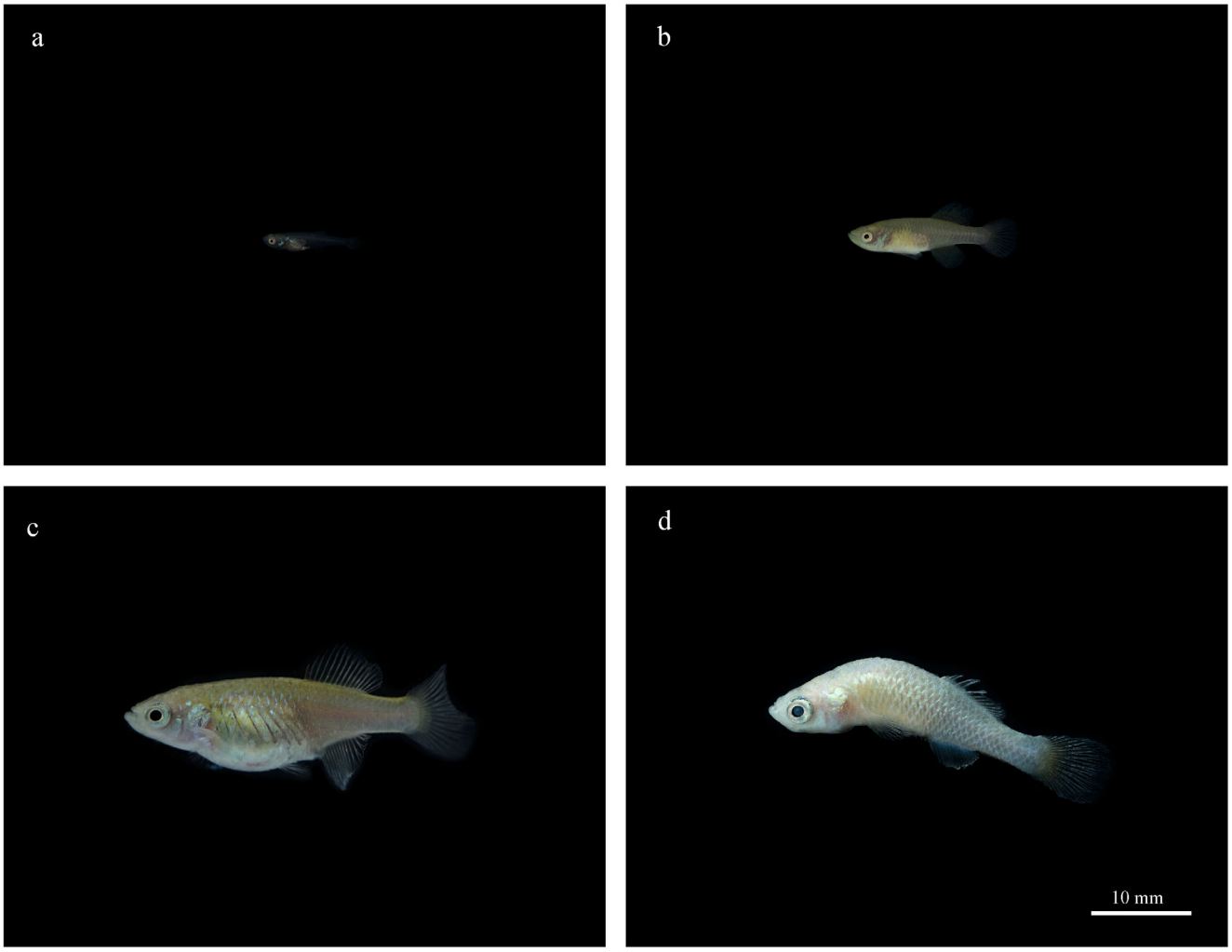

Fig. 5. *Nothobranchius guentheri* females of different age: a) 7 days; b) 2 weeks; c) 3 months; d) 18 months.

bled *de novo*, and annotated [67]. It was found that some genes were associated with lifespan and aging (*mtor*, *4ebp*, *s6k*, *insr*, *foxo3a/b*, *ampk*, and *polg*), similar association was demonstrated for several genes encoded in the mitochondrial genome [43]. Genetic loci associated with the lifespan in *N. furzeri* are located on the sex chromosome and include: 31 protein-coding genes, six genes of non-coding RNAs, and two genes of small nuclear RNAs. The following genes are worth mentioning: *GRN* gene encoding progranulin protein associated with neurodegenerative diseases and regulation of lifespan in mice; *GSTT1A*, glutathione-S-transferase gene, belonging to the redox enzymes involved in maintaining homeostasis that regulate lifespan in worms and humans; as well as genes *STAT3* and *STAT5.1*(*2of6*) coding for two transcription factors participating in regulation of inflammatory processes [43, 67].

Telomere shortening with aging, which is a typical process in the cells of humans and other organisms including model organisms [68-71], is not a biomarker of aging in Nothobranchius. It was demonstrated that telomere length shortened with age in the long-lived strain of *N. furzeri*, while they did not shorten in the GRZ strain likely due to the short lifespan of this strain [72]. However, the *N. furzeri* line with mutation in the telomerase gene exhibited premature infertility, sharp decrease of red and white blood cells, anomalies in the gut epithelia including decrease of polarity, and increase of the ratio between the size of nucleus and cytoplasm [73]. It seems likely that telomerase plays a key role in maintaining homeostasis in *N. furzeri*, but does not affect lifespan of the GRZ strain.

In many species, including humans, instability of mitochondrial DNA (mtDNA) is associated with aging [70, 74-77]. This is caused by the fact that mtDNAs do not have histones, and, hence, are more susceptible to mutagenesis [78]. Decrease in the number of copies of mtDNA with aging has been also observed [74, 79], which results in early manifestation of age-related diseases [80]. This process correlates with age in many tissues of *N. furzeri* including brain, liver, and muscles [81].

Age-dependent decrease of activity of proteasomes responsible for control of protein quality, which starts after puberty, results in the disruption of protein stoichiometry with aging. Progressive loss of normal proteostasis is considered as a main factor of neurodegeneration both in *N. furzeri* and in humans [82].

For what nosological categories use of Nothobranchius as a model of age-related disease is most suitable, valid, and promising? Neurodegeneration is the main physiological process investigated using fish of the *Nothobranchius* genus as a model of aging. For example, it was shown [4] that the glial fibrillar acidic protein GFAP becomes upregulated in the glial cell of the *N. furzeri* brain with age similarly to the process occurring in the aging mammals. This results in accumulation of lipid-rich pigment granules (lipofuscin) in the course of aging causing decrease of the neuronal support and protection provided by glia. At the same time, neurons degenerate as well, which is manifested by accumulation of beta-amyloid molecules in them followed by their aggregation and formation of plaques observed in the brain of patients with Alzheimer's disease – the most significant and known neurodegeneration disease in humans.

Age-related neurodegeneration of noradrenergic and dopaminergic neurons is observed in the aged *N. furzeri* resembling pre-symptomatic stage of Parkinson's disease [83]. This pattern of aging of the nervous system indicates that the natural genetic variations are capable of affecting susceptibility of dopaminergic neurons in *N. furzeri* and could be used for revealing modifying factors of age-related neurodegeneration.

Disruption of the functions of visual system is a valid sign of aging and manifestation of many neurodegenerative diseases. Moreover, specific markers of neurodegenerative processes have been identified in the retina of old *N. furzeri* [84].

Fish of the *Nothobranchius* genus are used as a model of osteoporosis associated with aging. Osteoporosis is a common age-related disease of the human musculoskeletal system. Cytological profile and mineral composition of bones in *N. furzeri* change with aging in a sex-dependent manner, which could be of importance during investigation of gender aspects of age-related diseases of musculoskeletal apparatus [4].

Hence, representatives of the *Nothobranchius* genus are perfect models for testing of not only potential geroprotectors [17, 85], but also of treatment strategies for age-related pathologies in humans.

CONCLUSION

Nothobranchius conceal a lot more mysteries, and in this review, we discussed only the most relevant issues, which could be explained at the current level of knowledge and technology. New and even more interesting topics will emerge with the developing of improved methodological approaches. What are the challenges on our path forward and what methodological problems are encountered by the researchers working with Nothobranchius?

The first group of methodological problems is related to peculiarities of animal housing and standardization of all manipulations with them. The experts know very well that it is not always possible to reproduce the results obtained and published by other esteemed laboratories. Standardization of the processes in collaboration with colleagues is of vital importance. For example, it is known that increase of water temperature increases average life expectancy. However, such attempts could have negative consequences. Designing of protocols that prevent the use of non-standardized materials, such as peat, resulted in the change of the ratio of diapauses in such laboratory animals as *N. furzeri* during embryogenesis – they practically did not have diapause III.

The second group of problems involves creation of the possibility for conducting non-invasive tests. This could provide the method for collecting biological material for the research not only post-mortem or by subjecting the organism to stress, and, hence, to obtain the dynamic data from the same animal. Currently attempts to use, for example, fin fragments, results in irreversible emergence of belly-sliders. Moreover, number of these cases increases with the age of animals, and fins are poorly regenerated in these cases.

And, finally, one of the least developed research areas from the methodology point of view is investigation of behavioral patterns of Nothobranchius. Cognitive-behavioral tests require simultaneously creativity, power of observation, attention to detail from the researcher, and, moreover, these tests are very time-consuming. However, this area of research is very appealing to researchers as it could provide many answers to the existing questions without the wait for new methodologies and instrumentation. Here are only few of those problems. In what directions the cognitive-behavioral tests should be optimized for different species and different age of animals? What could provide positive reinforcement during conducting cognitive-behavioral tests? In other words, how to design experiment without using only negative reinforcement, which, obviously, causes stress in the animal? How to solve the problem of the effect of different hierarchical status of the tested animals in the experiment? Or *vice versa*, how do design experiment that takes into consideration this status?

The aim of this review emphasizing potential of the model fish of the *Nothobranchius* genus is to stimulate the use of this model in the fight for long and healthy aging.

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tion of illustrations. All authors participated in analysis of the literature and writing the review.

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