Does *Nothobranchius furzeri* Gonarezhou die from a Parkinson's-like Disease?

Tyrone Genade
Ph.D. (Anatomy & Cell Biology), M.Sc. (Biochemistry)

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Summary

Neurodegeneration has diverse molecular causes. α-synucleinopathies are implicated in at least three neurodegenerative diseases: Parkinson's Disease, Dementia with Lewy bodies and Multiple System Atrophy. Age-related increases in α-synuclein and its oligomers have been observed in short-lived *Nothobranchius furzeri*. *N. furzeri* Gonarezhou, with a lifespan of 9–12 weeks, is a new model organism for aging research. With age the fish develops motor deficiencies that can be explained by the degeneration of its dopaminergic system. The author proposes to test the hypothesis that *N. furzeri* Gonarezhou are dying from an α-synucleinopathy, in particular: a Parkinson's-like Disease. Additionally, the effects of lifespan-altering interventions (calorie restriction, resveratrol and NT-020) on *N. furzeri* age-related neuropathology will be examined. NT-020 has been shown to reduce the α-synucleinopathy burden of aged *N. furzeri* as well as extend lifespan. Histological samples will be analyzed for the identification and localization of dopaminergic neurons as well as neuropathologies (α-synucleinopathies, neurofibrillary tangles, gliosis and β-amyloid plaques). The location and staging of the pathologies will be elucidated, as well as how the pattern of the pathology differs with age between the Gonarezhou and wild-derived strains. In addition, the effect of anti-aging treatments (e.g. NT-020) on pathology burden and pattern will be investigated. NT-020 is already recognized as a safe and cost-effective supplement that slows the progression of age-associated cognitive decline. This research will determine whether NT-020 may also find application in the treatment and prevention of human α-synucleinopathies.

Contents

1 Introduction

Neurodegeneration has diverse molecular causes, several of which are α-synucleinopathies: the aggregation and formation of cytotoxic α-synuclein protein oligomers in nervous system cells [1, 2]. α-synucleinopathies are implicated in at least three diseases: Parkinson's Disease (PD), Dementia with Lewy bodies (DLB) and Multiple System Atrophy (MSA) [1–3]; and are reported in 10 to 15% of dementia patient autopsies [4]. PD, DLB and MSA have a combined prevalence of 24.7 per 100 000 [5, 6], while the prevalence of Alzheimer's disease ranges between 2.4 and 127 per 100 000 [7] depending on age. Patients with PD have a median survival time of 15 years [8] compared to 3.4 to 8.5 years for Alzheimer's Disease [9]. The burden of α-synucleinopathies correlates with age [10] and may account for some of the motor and cognitive deficits in the elderly [11]. As α-synucleinopathies result in sizable financial and social burdens, over a long period of time, the role of α-synucleinopathies in the development and treatment of age-related pathologies is of critical importance.

1.1 *Nothobranchius* as a potential Parkinson's Disease model

Nothobranchius furzeri is being developed as a model organism for aging research [12, 13][1]. The original interest in this species was born from the observation that the Gonarezhou population of this species has a very short lifespan (9 to 12 weeks), which is amenable to experimental intervention [14, 15] (Figure 1). Analysis of wild-derived strains has confirmed the exceptionally short lifespan, but thus far the physiological and genetic cause (or causes) has not been determined [16]. Previous research by Kirschner et al. [17] had linked the Gonarezhou population's short lifespan to several chromosomal loci, one of which is

[1] The research implications for this fish have been realized by the popular press: http://www.nytimes.com/2015/03/03/science/in-short-lived-fish-secrets-to-aging.html?_r=0.

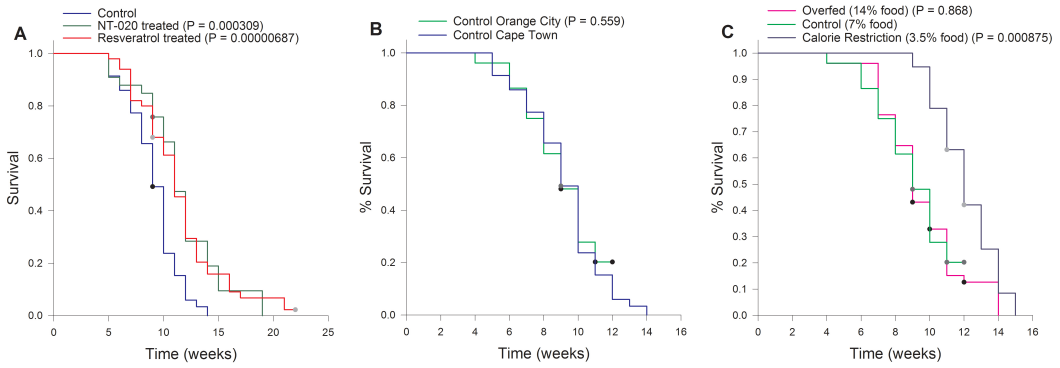

Figure 1: Comparative lifespans of *N. furzeri* Gonarezhou. A: Normal lifespan in Cape Town, South Africa as well as fish treated with Resveratrol and NT-020. B: A comparison of lifespans in Cape Town and Orange City, Iowa. C: Lifespan when fed a diet of varied calorie content. 14% food refers to 14 g of Repashy food formula "N. furzeri v 1.2" in a final volume 100 mL of 5% gelatin. P-values per graph are for comparison to the control group (Logrank test).

the *Park7* gene loci—a PD-linked gene. Mutations in this gene are responsible for autosomal recessive familial early onset PD [18].

As *N. furzeri* Gonarezhou age they manifest reduced spontaneous locomotion and swimming velocity as well as cognitive decline [15, 16]. The motor deficits manifested in PD and MSA are caused by the neurodegeneration of the substantia nigra. Fish do not have a substantia nigra [19] but instead have a series of dopaminergic neurons in the ventral diencephalon [20–22]. Studies in zebrafish [20, 21] and Medaka [22] wherein fish were given a PD-inducing toxin or mutated showed the same symptoms as naturally aged *N. furzeri* Gonarezhou. In the Medaka studies, the decline in spontaneous locomotion and swimming velocity were tracked to the middle diencephalic cluster of tyrosine-hydroxylase immunoreactive neurons [22, 23]. In addition, there was also signs of pathology in the dorsal motor nucleus (cranial nerve X nucleus) of the medulla—this is also observed in human PD staging [24]. The decrease in spontaneous locomotion and swimming velocity, as well as the decline in cognitive abilities of *N. furzeri* can be explained by damage to the dopaminergic system of the fish. If *N. furzeri* are indeed developing a Parkinson's-like Disease then they would be the first model organisms to develop it naturally, without genetic or toxocological intervention, making them a valuable tool in PD research.

Resveratrol treatment retarded the development of neurofibrillary tangles [15], a neurodegenerative pathology that is often concomitant with PD and DLB [2], as well as preserved motor and cognitive ability into old age. Fish use the cerebellum for cognitive processing [25]. In the olivopontocerebellar form of MSA the cerebellum degenerates [26] and could explain the cognitive decline of *N. furzeri* Gonarezhou; however, even in PD there is evidence for cerebellar degeneration and an decline in cognitive abilities [27]. Resveratrol has been shown to have neuroprotective properties in PD experimental models [28] and the neuroprotective effects of resveratrol on *N. furzeri* Gonarezhou could be due its ability to ameliorate α-synucleinopathy.

Supplementation with compound mixture NT-020 extended the lifespan of the fish as well as retarded the accumulation of the α-synuclein protein oligomers (unpublished data, Figure 2). The extent of the lifespan extension was no different to that of resveratrol

treatment. NT-020 is already recognized as a safe and cost-effective supplement, which slows the progression of age-associated decline in humans and animal models [29, 30] as well as having activities against neurodegeneration [31, 32]. Could NT-020 be effective in preventing or retarding α-synucleinopathy?

α-synuclein protein aggregates in MSA and PD cause dysfunction of the autonomic nervous system [2, 3]. Death of *N. furzeri* is often sudden (personal observation), implying a sudden catastrophic physiological failure. This sudden death can be preceded by loss of equilibrium (a motor deficit) or a period of gill hyperventilation and lethargy (autonomic failure). Some fish retreat from the shoal to languish in a corner of the tank (depression). Most individuals suffer such deaths between seven and 10 weeks of age[2]. These signs may potentially be explained by PD or MSA-like α-synucleinopathy. The author proposes to test the hypothesis that *N. furzeri* mortality is due to an α-synucleinopathy by characterization of the aging process of Gonarezhou and wild-derived strains.

1.2 Markers of α-synucleinopathies

N. furzeri possess genes for α-synuclein (unlike zebrafish), DJ-1 (Park7), tyrosine-hydroxylase and FOXA2 proteins. These proteins have high sequence homology to the human and Medaka fish (Table 1) and, except for FOXA2, are well researched in Medaka.

The development of Lewy bodies in neurons are diagnostic of α-synuclein [2]. This pathology is easily assessed by hematoxylin & eosin staining. For the visualization of Lewy neurites antibody probes against α-synuclein are required. Lewy bodies are expected to develop in catecholaminergic neurons of the fish nervous system. In Medaka, to which *Nothobranchius* are closely related, the catecholaminergic neurons were distributed in clusters from the telencephalon through to the diencephalon and in the medulla [22]. Lewy neurites should be visible in most brain structures, including the optic tectum based on

[2]This time frame varies with experimental location. In Pisa, Italy [15] and Cape Town, South Africa (unpublished data) median survival was 9–10 weeks while it was 11–12 in Jena, Germany [16]. It has not been determined what causes this variation.

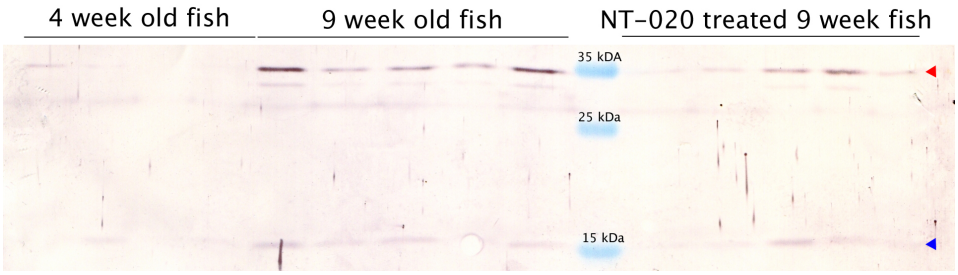

Figure 2: Anti-α-synuclein probed Western Blot developed by metal-intensified DAB. From left to right: four lanes of 4 week old *N. furzeri* brain protein homogenate, 5 lanes of 9 week old protein, protein marker lane (with band sizes in kDa) and 5 lanes of NT-020 treated 9 week old protein. Protein was first extracted in a buffer for soluble proteins, and then extracted a second time in a buffer to extract insoluble proteins. This second extraction is shown above, indicating that old fish have more α-synuclein protein aggregates. Aged fish have more of the cytotoxic α-synuclein oligomer (red triangle), visible at about 35 kDa. The α-synuclein monomer is visible at 15 kDa (blue triangle). The SNL-4 antibody of Virginia Lee was used [33].

Table 1: Amino acid sequence similarity between *N. furzeri* and that of human and Medaka proteins. The NFINtb *Nothobranchius furzeri* transcriptome browser [34] and UniProt http://www.uniprot.org/ were used to determine sequence homologies.

	Sequence similarity	
Protein	**Human**	**Medaka**
α-synuclein	61.0%	81.0%
tyrosine-hydroxylase	73.0%	86.0%
FOXA2	66.1%	96.0%
DJ-1 (Park7)	75.7%	91.0%

Medaka studies [35]. The burden of Lewy bodies and neurites is expected to increase with age in the *N. furzeri* Gonrezhou strain compared to age-matched controls of the wild-derived strains.

The *N. furzeri Park7* gene locus is linked to the short-lifespan of the Gonarezhou strain [17]. Relative to long-lived wild-derived strains, no mutations were found in the coding sequence of the Gonarezhou strain *Park7* gene suggesting that there might be a mutation in the regulatory elements. *Park7* codes for the DJ-1 protein. DJ-1 is reported to play a role as a redox sensitive chaperone, redox sensing protein and as a positive regulator of androgen receptor-dependent transcription. DJ-1 is hypothesized to function in a novel E3 ligase together with PARKIN and PINK1. *Park7* mutations result in either a loss of function or DJ-1 protein aggregation [36]. Up-regulating *Park7* expression was neuroprotective in animal models of PD [37]. It is expected that in *N. furzeri* Gonarezhou there would be a decrease in DJ-1 quantities in the soluble protein fraction of protein homogenates but an increase in total DJ-1 with age as the protein accumulates in Lewy bodies [38].

In the PARKIN/PINK1 double mutant Medaka there is a loss of both dopaminergic and noradrenergic neurons [23]. This loss of neurons was verified by a reduction of tyrosine-hydroxylase protein in protein extracts. Tyrosine-hydroxylase is an enzyme that is active in the biosynthesis of catecholamines (norepinephrine, dopamine, melatonin and neuromelanin) in the brain [39]. A reduction in tyrosine-hydroxylase protein is not simply indicative of a loss of dopaminergic neurons but of all catecholaminergic neurons of the brain. The FOXA2 protein is a specific marker of dopaminergic neuron [40]. It is expected that both tyrosine-hydroxylase and FOXA2 will decline with age in *N. furzeri* Gonarezhou as the burden of α-synucleinopathies increase with age and the catecholaminergic neurons die.

For the hypothesis that *N. furzeri* Gonarezhou is dying of a Parkinson's-like Disease to be confirmed the fish must develop Lewy bodies in a staged manner, and as the number of Lewy body bearing neurons die, the quantities of tyrosine-hydroxylase and FOXA2 in protein homogenates will decrease while the quanties of DJ-1 trapped in protein aggregates will increase proportionally with aggregated α-synuclein.

1.3 Experimental outline

To test the α-synucleinopathy hypothesis fish will be raised in the author's laboratory and the following experiments performed. Both the inbred Gonarezhou and long-lived wild-

derived stains will be used for the diagnosis of the α-synucleinopathy. The long-lived fish do not die suddenly [41] nor do they develop the same motor deficits [16]. The development and burden of α-synucleinopathy will be compared between the strains and it will be determined whether these correlate with the increase in mortality. Fish will be raised and some fish left to live out their natural lives while others will be killed for histological and protein analysis to detect changes in the expression of several proteins. *N. furzeri* brains will be sectioned and stained to determine the location of the dopaminergic and adrenergic neurons. Sections will also be stained for Lewy bodies. Criteria for distinguishing between the three forms of α-synucleinopathy are given by McCann et al. [2] and summarized in Table 2. In humans and mouse models of PD [24, 42, 43] there is evidence for extensive α-synucleinopathy in the nerves of the enteric nervous system (part of the autonomic nervous system) that spread into the dorsal motor nucleus of the vagus nerve, and then into the midbrain, diecephalon and telencephalon. *N. furzeri* Gonarezhou should show a similar pattern of staging while this pattern will be absent in wild-derived strains. Analysis of the digestive tract would help to exclude DLB. Only PD and MSA are known to manifest with α-synucleinopathies of the enteric nervous system. MSA can be differentiated from PD due to the occurrence of neurofibrillary tangles in PD while glial inclusions occur in MSA only. Analysis of protein extracts by Western Blot will be performed to correlate changes in cell pathology and number with changes in the levels of proteins associated with dopaminergic and adrenergic neurons.

N. furzeri Gonarezhou shows an accumulation of α-synuclein and its oligomers with age. It is possible that it is dying from a Parkinson's-like Disease. Its short lifespan can make it a valuable animal model in α-synucleinopathy research. There is already methods available to introduce novel mutations in *N. furzeri* [44]. In addition, several therapies are already available that extend its lifespan; and one of these, NT-020, is known to reduce the cellular burden of α-synuclein and its oligomers. If *N. furzeri* Gonarezhou can be shown

[3]This might not be true for fish that use the cerebellum for cognitive processing [25]. Previously reported evidence of dementia [15] could be evidence of fish cerebellar neurodegeneration.

Table 2: Summary of diagnostic criteria to distinguish between the three most common α-synucleinopathies. All information taken from McCann et al. [2] and Kim et al. [3] unless otherwise noted.

Disease	Parkinson's Disease	Dementia with Lewy bodies	Multiple System Atrophy
Motor signs	yes	some	yes
Dementia	late onset	early onset	none[3]
Pathologies			
Neuronal Cytoplasmic Inclusions (Lewy bodies)	yes	yes	yes
Neuronal Intranuclear Inclusions	no	no	yes
Glial Cytoplasmic Inclusions	no	no	yes
Dystrophic neurites	yes	yes	no
Amyloid Plaques	no	yes	no
Neurofibrillary tangles	yes	yes	no
Location	Spreads from Substantia Nigra & Enteric NS to Limbic System	Spreads from forebrain & does not reach Enteric NS	Present in Cerebellum, Pons & Olives; Striatonigral System; and Autonomic & Enteric Nervous Systems

to suffer from α-synucleinopathy that is treated by NT-020 then NT-020 will become of great therapeutic interest. Given its identity as a nutraceutical (dietary supplement), that has already shown value in slowing the progress of age-associated cognitive decline in humans [29], it could also serve as an economical over-the-counter preventative against the development of α-synucleinopathies in humans.

2 Methods & Experimental

2.1 Experimental

The following investigations will be performed:

- Inv. 1 Establishment of control populations of *N. furzeri* Gonarezhou and MZM 04-3 fish for acquisition of survival data and establishing of median survival times.
- Inv. 2 Optimization of fixation methods and demonstration of Lewy body α-synucleinopathy.
- Inv. 3 Validation of the α-synuclein, DJ-1, tyrosine-hydroxylase and FOXA2 antibodies.
- Inv. 4 Determination of the location of *N. furzeri* dopaminergic neurons.
- Inv. 5 Determination whether the three anti-aging interventions (Calorie Restriction, Resveratrol and NT-020 treatment) all similarly affect α-synucleinopathies in the Gonarezhoul population; and corroborate histological changes with the measurement of protein expression by Western Blot.
- Inv. 6 Determination of the frequency and distribution of α-synucleinopathies by antibody labeling in young and old fish of the Gonarezhou and MZM 04-3 populations of *N. furzeri*; and corroborate histological changes with the measurement of protein expression by Western Blot. Use Thioflavin-S and Congo red to examine brains for other pathologies.
- Inv. 7 Validate the tau, β-amyloid and AIF1 antibodies and staining methods for histology and Western Blot.
- Inv. 8 Using antibodies, determine the frequency and distribution of neurofibrillary tangles and β-amyloid plaques as well as neuroinflammation.
- Inv. 9 Validate the CNPase and Neuron Specific Enolase antibodies for histology and Western Blot.
- Inv. 10 Determine whether α-synucleinopathy corresponds with neuronal or glial marker expression; and in which tissues and brain regions to determine which of the human α-synucleinopathies best correlates with the fish pattern of pathology.

The research will be performed in stages. Animal experiments will be performed first together with the optimization of the fixationn protocols, validation of the antibodies (Inv. 1–5). Following the success of these experiments Inv. 6–8 will begin. At this point there should be evidence for or against glial inclusions based on the cellular morphology of α-synucleinopathy containing cells. If warranted, further experimentation with glial markers will proceed (Inv. 9). Finally, all the data will be compared to Table 2 and conclusions

drawn as to whether or not the fish are dying from an α-synucleinopathy and whether it correlates with one of the human conditions as well as how effective the experimental interventions (Inv. 4) were at preventing the pathology. The experimental aspect of this research project is expected to take three years. Year 1 will cover Inv. 1–5; year 2 those Inv. 6–8; and year 3 with Inv 9–10. All data should be processed and written up for publication by the end of year 4.

2.2 Animal experiments

Two strains will be reared in the laboratory: the short-lived *N. furzeri* Gonarezhou and long-lived, wild-derived MZM 04-3. Fish will be hatched, raised and maintained to old-age and death according to the standard protocol [45] with the single modification of using a Repashy Gel food, formula N. furzeri v 1.1, at 7 g/100 mL 5% gelatin (final volume)[4]. Multiple experimental groups, each consisting of 10 to 12 fish in a recirculating aquarium system, will be established and used to obtain survival data and samples for analysis. Experimental replicates will be performed either simultaneously or in sequence depending on aquarium space. The number of fish needed to gather statistically meaningful data will be determined empirically, and a breeding population maintained to sustain the colony. All fish will be used to gather survival for control or experimental groups. Fish will be anesthetized using MS 222 (100 mg/L) before immersion in ice-water and decapitated.

Tissue and protein samples will be taken at 4-weeks-old (young fish) and 9-weeks-old (aged fish, median-survival-point for the Gonarezhou population). Further samples of the MZM 04-3 population will be taken at their median-survival-time. Tissue will be processed as described in Section 2.4 and protein extracted as described in Section 2.3.

In addition to standard feeding regimes, the following modified treatments will be administered:

- Calorie-restriction group (3.5 g/100 mL 5% gelatin)
- Resveratrol-treatment group (7 g/100 mL 5% gelatin with 120 μg resveratrol/mL) [15]
- NT-020-treatment group (7 g/100 mL 5% gelatin with 263 μg NT-020/mL) (Figure 1)

Experimental fish will be left to live out their natural lifespans until lifespan extension is confirmed whereafter the 9-week-old samples will be obtained from subsequent experimental groups.

Additional samples, taken at 5, 6, 7 and 8 weeks of age, may be needed to determine disease progression and the staging of the neuropathies.

Ethics approval has been obtained from the Ethic Committee of Northwestern College, Iowa.

[4] In experiments performed in the laboratory the food dose was optimized to a level where it did not affect lifespan (Figure 1, unpublished data). Limits of food:gel ratio at which signs of calorie restriction manifest, as well as at what levels signs of over-feeding manifest were determined. The food formula is available at http://www.store.repashy.com/test/index.php?dispatch=products.view&product_id=610

2.3 Protein extraction & analysis

Protein extracts of brain and digestive tract tissues will be examined for changes in protein expression of AIF1, α-synuclein, β-amyloid, FOXA2, GFAP, neuron specific enolase, PARK7, phosphorylated tau and tyrosine-hydroxylase. The antibodies in Table 3 will be used to detect relative increases or decreases in protein between young, old and treated fish, as in Figure 2. Proteins will be extracted as previously described [46, 47] and processed by SDS-PAGE. TGX Stain-Free FastCast of Biorad will be used for SDS-PAGE electrophoresis and total protein be used as loading control [48] and then 0.2 micron nitrocellulose membranes for Western Blotting. Membranes will be blocked using 2% PVP-40 [49] or 5% milk and visualized using Biorad Clarity or metal intensified DAB.

Data will be analyzed using Image-J and SigmaPlot's ANOVA functions followed by post hoc tests for statistical significance.

2.4 Histology

Fish tissues will be fixed in decalcifying fixatives: Bouin's Solution [19], Davidson's Fixative or 10% formalin [50] and then decalcified in Kristensen's Fluid or 14% EDTA and processed for wax impregnation. Tissue will be serially sectioned at 6 μm. Fixation quality and the presence of Lewy bodies will be assessed by Hematoxylin & Eosin staining. The best fixative protocol will be used for immunohistochemistry methods outlined below.

Sections will be processed for immunohistochemistry by the standard protocols [50].

Table 3: List of antibodies to be employed in the project. Antibodies that need to be purchased are indicated with an *. All antibodies have either had their immunogen sequence compared to *N. furzeri* protein sequences or been tested on the closely-related Medaka fish.

Marker	Antibody	Manufacturer	Host	Cellular target
visualization of Lewy bodies and dystrophic neurites	SNL-4 anti-α-synuclein	custom	rabbit	α-synuclein
	*Synuclein-alpha nitrated Antibody Syn 505	Life Technologies	mouse	oxidized α-synuclein
	*Clone No. pSyn #64 anti-synulcein	Wako	mouse	detects aggregated α-synuclein
	*Medaka anti-α-synuclein	custom	rabbit	fish specific α-synuclein
label of DJ-1	*anti-PARK7/DJ1	Abcam	goat	DJ1 in cells
label catecholaminergic neurons	*anti-Tyrosine Hydroxylase	Millipore	mouse	Tyrosine Hydroxylase in neurons
label dopaminergic neurons	*anti-FOXA2	Abcam	goat	FOXA2 in neuronal nuclei
neurofibrillary tangles form in neuronal cell bodies in the course of neurodegeneration	*anti-Phospho-PHF-tau pSer202/Thr205, Clone: AT8	Thermo	mouse	tau protein in neurons, neurites & axons
β-Amyloid plaques	*anti-β-Amyloid	LSBio	goat	β-Amyloid plaques
gliosis & astroglial marker	anti-GFAP, GA5	Sigma	mouse	glial fibrillary acid protein in astroglia
used as a marker of neurons and axons	*anti-Neuron Specific Enolase	Santa Cruz	goat	neuron specific enolase in neurons
marker of myelinating cells	*anti-CNPase	Sigma	mouse	oligodendrocytes
up-regulated in the course of neurodegeneration	anti-Iba1 (AIF1)	Abcam	goat	microglia/monocytes
leukocytes	anti-L-plastin	custom	rabbit	pan-leukocyte marker

Briefly, the slides will be heated, the wax melted and removed in two changes of xylene before moving to water. Sections will then be soaked 10 minutes in 70% ethanol with lithium carbonate to remove picric acid (due to Bouin's fixation) before moving to water. Proteinase K antigen retrieval will be used to demonstrate α-synucleinopathies [50]. This method does not interfere with the demonstration of neurofibrillary tangles and plaques [51]. Antibodies and their respective targets are profiled in Table 3. Additional antigen retrieval steps may be required for the employment of certain antibodies.

It is expected that Lewy bodies will be found most abundantly in catecholaminergic neurons. Sections will be examined by eye for the presence of neuromelanin, a marker of catecholaminergic neurons [52]. The tyrosine-hydroxylase antibody will also be used to label catecholaminergic neurons in the brain of *N. furzeri* and the FOX2A antibody to distinguish dopaminergic neuron nuclei.

Four pathologies will be studied: Lewy bodies, gliosis, neurofibrillary tangles and β-amyloid plaques. Plaques and neurofibrillary tangles (in Alzheimer's Disease and Frontotemporal Dementia) [53] and α-synucleinopathies have distinct origins and staging patterns [24, 54]. Whole brains, serially sectioned, will be examined to determine the spread of the disease at each particular age. Gliosis is a general marker of neurodegeneration and will be detected by a comparative increase in GFAP protein in the astroglia.

To identify the four pathologies tissue sections will be double or triple labeled. The α-synulcein antibodies will be incubated on tissue sections (and visualized with metal intensified DAB) to visualize α-synucleinopathies. Thioflavin-S can be used to visualized β-amyloid plaques and neurofibrillary tangles. Alternatively, sections can be labeled for α-synuclein and neurofibrillary tangles using antibodies (anti-phospho-PHF-tau, Table 3) and then stained with Congo Red to visualize β-amyloid plaques. The antibody labeled structures would be visualized by a complimentary combination of Horse Radish Peroxidase and Alkaline-Phosphatase coupled secondary antibodies together with the appropriate chomogen (e.g. metal intensified DAB and Abcam StayRed respectively) followed by fluorescent excitation of the Thioflavin-S or Congo Red. Images of fluorescent labeled plaques laid over light microscopy images of chromogen developed structures have successfully been used to demonstrate α-synucleinopathies together with plaques and tangles [55]. The suitability of Thioflavin-S or Congo Red for imaging will be assessed empirically. A similar labeling strategy will be used to determine whether the α-synucleinopathy is in a neuronal cell (labeled with neuron-specific enolase), a glial cell (anti-GFAP for astroglia and anti-CNPase for oligodendrocytes) or an immune cell (anti-AIF1 or anti-L-plastin).

The nature of the α-synucleinopathy will be assessed using the diagnostic criteria shown in Table 2. In brief: anti-α-synuclein immunoreactivity in hematoxylin stained nuclei, as well as anti-α-synuclein immunoreactivity in glia, will be interpreted as evidence for MSA. The presence of neurofibrillary tangles would count as evidence against MSA but evidence for PD or DLB. The presence of neurofibrillary tangles without β-amyloid plaques would be indicative of PD.

Data will be analyzed using criteria of the DLB consortium [56] and statistical analysis performed with SigmaPlot.

3 Research Significance

The research will confirm the presence or absence of α-synucleinopathies in *N. furzeri* as well as provide information that can be used to determine the nature of the disease through further experimentation. Should *N. furzeri* be shown to develop α-synucleinopathies it can become a powerful research tool into diseases such as PD, DLB and/or MSA that currently have to rely on genetically engineered or toxin-induced rodent and fish models with far longer lifespans. *N. furzeri* experimentation can be used to fast-track drug-screening on animal models. While resveratrol has already been the focus of much research as a therapeutic for PD there has been little investigation into the usefulness of NT-020 for treating (or preventing) PD, despite its known effectiveness in treating other neuropathologies [29–32]. NT-020 is free of side-effects and readily available as a nutraceutical. It could serve as an economical preventive medicine for those with genetic predisposition to α-synucleinopathies such as PD.

Several research outputs are expected from this research.

1. A publication on α-synuclein tissue expression in *N. furzeri* and its correlation with age in the Gonarezhou and MZM 04-3 populations.

2. A neuropathological study on the development and distribution of α-synucleinopathies, plaques and tangles.

3. An assessment on the staging of neuropathologies as well as the determination of the type of α-synucleinopathy affecting the fish.

4. Publication of the effects of Calorie Restriction, resveratrol and NT-020 on α-synuclein and tau expression, as well as the incidence of α-synucleinopathy, plaques and tauopathy in *N. furzeri* Gonarezhou.

5. Several neuro-anatomical studies concerning the expression pattern of CNPase, neuron-specific enolase and immune cell markers (L-plastin and Iba1).

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A Biographical Sketch

The author obtained a Ph.D. in Anatomy and Cell Biology for his research into the aging of *N. guentheri* [57]. He is skilled in execution of *Nothobranchius* experimentation and methods of protein and tissue analysis. He has published several articles, the most recent (from 2014) has been cited 15 times (Google Scholar) and his initial work on *N. furzeri* and resveratrol (2006) has been cited more than 500 times. The author also possesses a M.Sc. in Biochemistry.

He lectures Anatomy and Physiology (as well as introductory Biology) at Northwestern College, Iowa, as an tenure-track Assistant Professor.

He currently collaborates with the laboratory of Prof de Girolamo and Livia DeAngelo of the Facoltá di Medicina Veterinaria of the University of Naples Federico II and is completing experiments begun with the Ph.D. student, Alessia Montesano of the aforementioned institution.

B Curriculum Vitae

Personal Information

Citizenship: South African
Address: 416 5th St NE, Orange City, Iowa, 51041
Telephone: 712-230-4101 (mobile)
Email: tyrone.genade@nwciowa.edu (work), tgenade@gmail.com (personal)

Current Employment

Assistant Professor of Biology at Northwestern College, Iowa. Teaching: Anatomy, Physiology & Biology.

Highest Qualifications

• Ph.D. Anatomy & Cell Biology, 2012, University of Cape Town. Subject: neurobiology of aging and anti-aging.
• M.Sc. (Biochemistry), University of Stellenbosch. Subject: computer modeling of steroid metabolism.

Research Outputs

Montesano A, Arcamone N, **Genade T**, De Girolamo P (2015) Central regulation of food intake during aging in the teleost fish Nothobranchius furzeri. X Congress of the Nazionale Associazione Italiana dei Morfologi Veterinari.

Genade, T. & Cor De Wit (2014) What light do plants need and how can we choose a good lamp? Aquatic Plants South Africa Forum. http://www.apsa.co.za/xenforo/threads/what-light-do-plants-need-and-how-can-we-choose-a-good-lamp.11620/[5]

[5]These articles were mirrored at http://www.iowaaquatichobbyist.com/viewtopic.php?f=17&t=2793 on The Iowa Aquatic Hobbyist forum.

Genade, T. (2014) Lighting basics for people new to the Aquascaping hobby. Aquatic Plants South Africa Forum. http://www.apsa.co.za/xenforo/threads/lighting-basics-for-people-new-to-the-aquascaping-hobby.11329/[5]

Genade, T. and Lang, D. M. (2013) Resveratrol extends lifespan and preserves glia but not neurons of the *Nothobranchius guentheri* optic tectum. Experimental Gerontology. Exp Gerontol. 48:202-12. doi: 10.1016/j.exger.2012.11.013. Epub 2012 Dec 7. Featured on Global Medical Discovery: http://globalmedicaldiscovery.com/; and on Labome.org: http://www.labome.org//exp/genade/t-genade-2188439.html. Citations: 15.

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Valenzano, D.R.; Terzibasi, E.; **Genade, T.**; Cattaneo, A.; Domenici, L. and Cellerino, A. (2006) Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. *Current Biology* 16(3):296–300. Citations: 547.

Genade, T.; Benedetti, M.; Roncaglia, P.; Cattaneo, A. & Cellerino. (2005) A. Annual fishes of the genus Nothobranchius as a model system for ageing research. *Aging Cell* 4:223–233. Citations: 96.

Poster presentations or work presented at conferences/symposiums

Resveratrol extends lifespan but does not prevent aging-related neuron loss in Nothobranchius guentheri. Poster presented at the CBBRe Annual Research Symposium, August 21–22, 2014.

Resveratrol extends lifespan but does not prevent aging-related neuron loss in Nothobranchius guentheri. Poster presented at the Annual Congress of the Neurological Association of South Africa, March 2013.

A fishy story of aging, neurodegeneration and resveratrol: how Nothobranchius *fish can expedite research into cognitive aging* Seminar present at the The Albertina and Walter Sisulu Institute of Ageing, Groote Schuur Hospital/University of Cape Town, November 2012.

Resveratrol extends lifespan but does not prevent aging-related neuron loss in Nothobranchius guentheri. Nanosymposium talk (abstract 826.07) at the 201s Society for Neuroscience meeting in New Orleans.

Aging of plasticity related CNS features and resveratrol neuroprotection in Nothobranchius guentheri. Poster Presentation at the 2011 Society for Neuroscience meeting in Washington DC.

A new animal model of age-dependent cognitive deficit. Oral communication by D.R. Valenzano, at the International Symposium for Neuroplasticity, Neurotrophic Factors and Mood Disorders, Pisa Italy. April 8–9 2005.