APPLICATION TO AMEND THE LIST OF SPECIMENS SUITABLE FOR LIVE IMPORT

Draft Risk Assessment Report

1. Taxonomy of the species

a. Family name: Aplocheilidae

b. Genus name: Nothobranchius

c. Species: *N. furzeri*

d. Subspecies: No

e. Taxonomic Reference: http://www.uniprot.org/taxonomy/105023

f. Common Names: Turquoise killifish

g. Is the species a genetically-modified organism (GMO)? No

2. Status of the species under CITES

Species is not listed in the CITES Appendices.

3. Ecology of the species

a. Longevity: what is the average lifespan of the species in the wild and in captivity?

The average lifespan of the species in captivity is 13 weeks (1). Wild derived *N. furzeri* from three different habitats showed a maximum lifespan of 25-32 weeks (1).

b. What is the maximum length and weight that the species attains? Provide information on the size and weight range for males and females of the species.

As per the original formal description of the species, the standard length of *N. furzeri* from a wild population ranged from 2.5cm to 4.4cm (2). The maximum length the species can attain is 7cm (3). The mean maximal body weight reported for males is 3.8 ± 1.1 g (range: 0.5 – 6.7 g) and 2.2 ± 0.6 g (range: 0.2 - 3.6 g) for females (4). Growth rates of Nothobranchius in the wild have been reported to be similar to those in captivity (5).

c. Discuss the identification of the individuals in this species, including if the sexes of the species are readily distinguishable, and if the species is difficult to distinguish from other species.

N. furzeri are highly sexually dimorphic and dichromatic; males are robust and colourful (yellow or red) while females are dull. Female *N. furzeri* are always smaller than males, their fins are translucent and body is pale brown (3). These features are evident in the representative photographs below:

Representative images of a yellow form, male (a); red form, male (b); adult female (c) *N. furzeri.*

Reprinted by permission from Macmillan Publishers Ltd: Nature Protocols; Polačik et al., 2016, copyright (2016) – See Agreement at end of application

d. Natural geographic range. What is the country of origin and what is the natural distribution of this species? Where does the species occur naturally? Exclude any areas where the species has been introduced through human intervention. Describe any population limiting influences in its natural range including: predator/prey relationships, competition, availability of resources etc.

The N.furzeri require tropical climate and soft substrates for successful breeding. The species was first reported in the Sazale Pan (Gonarezhou National Park), Zimbabwe in 1968 (2). Since then the species has been reported to encompass the area between the Save river in Zimbabwe, the Lebombo ridge in South Africa and Limpopo River in Mozambique (1, 6).

Environmental predictors of species presence has recently been described. The presence of *N. furzeri* is most significantly related to altitude, with populations absent from coastal areas (no occurrence below 24 m above sea level) and rarely recorded at high altitude. *N. furzeri* is more common at sites with a soft substrate and positively associated with littoral, submerged and Nymphaea vegetation. *N. furzeri* is resides in temporary pools and lays eggs in muddy bottoms. However, in order to survive, *N. furzeri* eggs require a very specific type of substrate, composed of vertisol developed over alluvial deposits, to survive. Brian Watters examined more than a thousand Nothobranchius habitats as well as pools that do not host Nothobranchius fishes. In all cases, the reasons for the presence or absence of Nothobranchius were primarily determined by the nature of the substrate. Without the particular type of substrate (alluvial vertisols) no eggs can survive the dry season (6-8).

e. Is the species migratory?

No.

f. Does the species have the ability to hibernate in winter or aestivate (go into stasis or torpor) in the summer months)?

No, but as a result of routine drying of their environment, *N. furzeri* eggs are desiccation-resistant and can remain dormant in the dry mud for one and maybe more years by entering into developmental arrest (7).

g. Does the species have the ability to breathe atmospheric air i.e. has accessory breathing organs? (fish and other mobile aquatic animals)

No.

h. Outline the habitat requirements for all life stages of the species:

- physical parameters (e.g. salinity, oxygen, pH, temperature) of the natural habitat;

  N. furzeri is a freshwater species that resides in temporary pools. The water pH range 6.5 - 7.0; degree of hardness ranges from 4 - 15dH; and temperature ranges from 23°C - 30°C, consistent with a tropical climate (3, 9).

- What nest sites can the species use? 'Nest' is taken to mean a specific area individuals return to in order to sleep, bear or rear young. Identify where the species does/can nest. For example tree hollows; burrows; caves; buildings; cliff faces; dams, lake, pond marsh, swamp, reed-bed; particular ground surface; particular vegetation type; other (specify).

  N. furzeri are bottom spawners and require soft substrate for successful breeding. Before their seasonal habitat disappears adults spawn and lay eggs in the muddy bottom. Embryos survive encased in dry mud in a dormant state and hatch at the onset of the next rainy season to repeat the life cycle.

  In captivity, *N. furzeri* nest on peat or fine silica sand (7, 8).

- Does the species nest, shelter or feed in or around any of the following habitats? Marshes or swamps; estuaries, lakes, ponds or dams, rivers, channels or streams, banks of water bodies; coastal beaches or sand dunes (specify). This question seeks to identify if the species could impact on habitat(s) listed.

  Yes. *N. furzeri* is resides in temporary pools and lays eggs in muddy bottoms. However, in order to survive, *N. furzeri* eggs require a very specific type of substrate, composed of vertisols developed over alluvial deposits, to survive. Brian Watters examined more than a thousand *Nothobranchius* habitats as well as pools that do not host Nothobranchius fishes. In all cases, the reasons for the presence or absence of Nothobranchius were primarily determined by the

nature of the substrate. Without the particular type of substrate (alluvial vertisols) no eggs can survive the dry season (6-8).

i. Social behaviour or groupings. Describe how the animal would naturally dwell, for example in social groups, pairs, solitary; animals may be predominantly solitary except during breeding seasons etc. How does the species behave towards its own kind and other species?

N. furzeri are naturally found in social groups, which are often dominated by females (10). Male *N. furzeri* can get aggressive towards other *N. furzeri* males as they compete for access to females for successful breeding (11). Several Nothobranchius species often inhabit the same pools, and *N. furzeri* has not been reported to be selectively aggressive to other species (12).

j. Is this species ever territorial or does it exhibit aggressive behaviour?

The occurrence of multiple Nothobranchius species, including *N. furzeri*, in the same pools in the natural habitat suggests that the latter is not a territorial species (12). Although *N. furzeri* males attack other Nothobranchius males during breeding season, they do not defend a territory (11) .

k. Characteristics that may cause harm to humans or any other species.

None reported in the studies that have examined *N. furzeri* in the wild. The *Nothobranchius* species are not known as piscine predators in the wild (10, 12) or in captivity (3).

4. Reproductive biology of the species

a. At what age does this species reach sexual maturity (males and females)?

3-4 weeks after hatching (5).

b. Discuss the species' ability to reproduce; triggers for breeding; breeding site requirements.

Male spawning behaviour is simple and brief with males intercepting passing females and immediately attempting to spawn with them with no elaborate mating display (11). *N. furzeri* are bottom spawners. Eggs are laid in the sediment and the jerking movements during oviposition likely help to cover the eggs with sediment, However, the presence of a substrate is not required for oviposition and there are no specific requirements for spawning substrate (6). See comments above on specific requirements of sediment/bedding for embryos that is necessary for breeding

c. How frequently does breeding occur?

After reaching sexual maturity they reproduce daily, with 20–40 single eggs laid each day in multiple spawning acts (13).

d. For sessile aquatic invertebrates include details of:

- the length of time spent as motile larvae or plankton
- growth patterns (e.g. is it colonial or does it grow as a solitary animal) characteristics or behaviour that enable the species to survive drought, or other adverse conditions (e.g. forming cysts or spores).

Embryos survive encased in dry mud in a dormant state for at least a year

e. Can individuals of the species change sex? (reptiles, amphibians, fish and other mobile aquatic animals)

No.

f. Ability of the species to hybridise. Describe any known crosses. Are progeny of such crosses fertile?

Mating between *N. furzeri* and N. orthonotus has been previously described. Mating between *N. furzeri* males and N. orthonotus females was absent under standard experimental conditions and eggs were not viable when fish were forced to mate in a modified experimental setup. In contrast, male N. orthonotus indiscriminately mated with *N. furzeri* females, the eggs were viable, and offspring successfully hatched. Most spawnings, however, were achieved by male coercion and egg production and embryo survival were low (11).

g. Could the species hybridise with any Australian native species? Identify whether the species could negatively impact native species through hybridisation (cross-breeding with native species).

The only *N. furzeri* hybrid cross reported to date is that with N. orthonotus (11). Whilst female *N. furzeri* were successful in mating with male N. orthonotus females, the eggs survival was found to be low (11). With this data it can be suggested that *N. furzeri* could potentially hybridise with other native species but the viability of progeny would be low and hence would not have any negative impacts.

h. Are individuals single sexed? (i.e. either male or female) or hermaphroditic (i.e. have both male and female reproductive organs).

Individuals are single sexed, male or female, and females do not store sperm (13).

5. Established feral populations

a. Has this species ever established a breeding population outside of its native range? Identify any areas where this species has established a breeding population outside of its natural range.

No. Attempts to relocate Nothobranchius in temporary habitats where no native Nothobranchius are present always failed to generate stable colonies (14). The

inability of Nothobranchius to establish a breeding population outside its normal range is supported by the systematic study that showed that presence/absence of *N. furzeri* is strongly affected by its habitat characteristics (12). In particular, the requirement for suitable substrate for embryos seems restrictive for the species (6-8).

b. Is the species considered a pest anywhere in its natural or introduced range? A pest is a species of animal that causes wide-scale economic cost or amenity loss through its presence or activities. Identify whether this species is subject to active management to reduce population numbers.

   No. The species is not considered a pest anywhere in its natural or introduced range and it is not subject to active management to reduce population numbers.

c. Has the species been introduced to other countries, even if it has not established feral populations?

   N. furzeri eggs have been introduced into the following countries: USA, Germany (7), Italy.

6. Environmental risk assessments of the species

Have any risk assessments of the species, or similar species been carried out in Australia or overseas? Include the results of those assessments in the report.

A risk assessment report for the import and keeping of exotic freshwater and estuarine finfish has been produced by the Australian Bureau of Rural Sciences in 2004. In this report, it shows that the 2 attempts were made worldwide to introduce Nothobranchius to a different environment, both of which were unsuccessful (15). As a result, no genus risk score has been determined. No other risk assessment reports on *N. furzeri* are currently available.

7. Likelihood that the species could establish a breeding population in Australia

a. Ability to find food sources. Is the species a generalist feeder or does it have specific food needs? What is the likelihood of it finding food in Australia if it was released or escaped? Describe the feeding characteristics of the species, including whether it has a similar diet to any Australian native species.

   N. furzeri is a generalist predator and feeds on a variety of small aquatic invertebrates with a preference for small crustaceans (Cladocera, Copepoda, Ostracoda and Conchostraca) (16). Given that it undergoes rapid ageing, it requires large quantities of food in a short time. This may hinder its survival in the event it escaped or was released.

b. Ability to survive and adapt to climatic conditions. Describe the characteristics or behaviour that would enhance its ability to survive extreme climatic conditions (e.g. drought) and its ability to adapt to different environments.

Eggs of *N. furzeri* can undergo a state of dormancy making them tolerant to extreme climatic conditions such as drought (6). However, the eggs need to hatch in a very precise time window. Prolonged incubation due to unfavorable climatic conditions, and subsequent failure to hatch, will exhaust their energy reserves resulting in their death (3, 6). Adult *N. furzeri* on the other hand cannot survive extreme climatic conditions, which will prevent them from establishing a breeding population in Australia (3, 17).

c. Ability to find shelter. Can the species live in modified habitats? Identify if this species can live in habitats that have been modified by humans, either directly or indirectly, e.g. plantation forests; gardens; orchards; vineyards; crops; cities or towns; buildings; improved pastures; dams, channels or drains; other (please specify).

No. Modified environments would have little or no impact on required habitat for the species. As mentioned in 7(b) *N. furzeri* have a very specialized life cycle and live in a very specialized environment (6, 12) which will limit their survival in Australia.

d. Reproduction. Could factors such as longevity, birth rates and numbers of offspring increase the likelihood of the species to establish?

No. The short life span and peculiar life-cycle of *N. furzeri* (6) will limit the establishment of the species in Australia.

e. Are there any limiting influences on the species' natural range? Predator/prey relationships, competition, availability of resources etc. Assess what similar population constraints might exist in Australia.

Ambient temperature, aridity, food rations and availability of suitable breeding grounds (soft muddy substratum composed mainly of vertisol soil and turbid water) will all significantly affect growth, reproduction and lifespan of *N. furzeri* hence, limiting its natural range (6).

Additionally it has recently been show that *N. furzeri* have strict geographical requirements: The presence of *N. furzeri* was most significantly related to altitude with populations absent from coastal areas (no occurrence below 24 m above sea level) and rarely recorded at high altitude. *N. furzeri* was more common at sites with a soft substrate and positively associated with littoral, submerged and Nymphaea vegetation (12).

f. Address the issue of increased potential for feral population establishment if more individuals of the species were present in Australia.

There are several *Nothobranchius* species already existing in Australia and despite this there is no feral population established to date. The conditions in Australia, such

as appropriate soil availability and climate, are suboptimal for *N. furzeri* making it highly unlikely that a feral population will be established.

8. Provide a comprehensive assessment of the potential impact of the species should it become established in Australia

a. Does the species have similar niche/living requirements to native species?

- Could wild populations of the species use the same resources as native Australian species, for example that it would compete with for food, shelter etc.

  No. The presence of *N. furzeri* was most significantly related to altitude with populations absent from coastal areas and rarely recorded at high altitude. *N. furzeri* is most common at sites with a soft substrate and positively associated with littoral, submerged and Nymphaea vegetation (12).

b. Is the species susceptible to, or capable of transmitting any pests or diseases?

  Yes. In the wild *Nothobranchius* serve as an intermediate host for several species of internal parasites such as flukes (metacercariae), which are the most common, nematodes, and larval cestodes, with the definitive hosts being waterbirds that need to consume *Nothobranchius* to be infected (3).

c. Probable prey/food sources

- Does the species attack or prey on wildlife? Identify if the species has the capacity to attack or prey on wildlife. If 'yes', specify whether the prey are: waders or waterfowl; other birds; mammals < 1 kg; mammals 1–5 kg mammals > 5 kg; amphibians; vertebrate eggs; fish; aquatic invertebrates; reptiles; insects; land invertebrates; other; (specify).

  Yes. *N. furzeri* feeds on small aquatic invertebrates (6).

- Does the species attack or prey on domestic or commercial animals or plants?

  No

d. Impacts on habitat and local environments.

- Could the species reduce the ground vegetation cover to an extent where it could cause or increase soil erosion? This question looks at identifying if the species, through feeding, digging or other activities could have a detrimental impact on vegetation such that the underlying soil is exposed to increased erosion.

  No. In over 200 *N. furzeri* habitats studied, there has been no report of detrimental effects of the species' existence on the vegetation or on the

underlying soils (10, 12).

- Does the species construct burrows or dig near or around waterways? Identify if the species does/can burrow or otherwise disturb the substrate (soil or sand) around waterways.

  N. furzeri do not construct burrows but egg spawning is associated with subtle disturbance of the sediment, which is most likely to help cover the eggs with sediment (6).

- Has the species ever been recorded causing damage to: native animals' habitats; natural communities; native plants; forestry; agriculture?

  No. Studies examining *N. furzeri* habitats have reported no damage inflicted by the species to native animals' habitats, natural comminutes or plants (10, 12).

- Could the species inhibit tree seedling regeneration in forests and woodland? This question aims to identify if the species could have a negative impact on regeneration in native forests and woodlands.

  No. The species is not found in forest or woodland areas and as such does not inhibit tree seedling regeneration In these areas (10, 12).

- Could the species spread weeds? Identify whether the species could spread weeds through carrying seeds on their fur/feathers, defecating the seeds at a distance from the parent plant or moving viable vegetative matter to new areas.

  No. Areas inhabited by *N. furzeri* have not been reported to have an increased prevalence of weeds, suggesting that *N. furzeri* do not spread them (10, 12).

e. Discuss any control/ eradication programs that could be applied in Australia if the species escaped or were released. Are any such eradication programs already available in Australia?

   N. furzeri have a unique life cycle and biology and have specific requirements of optimal temperature, aridity, food rations and availability of suitable housing. All these factors will restrict their survival in the wild making the risk of their potential escape negligible (6) (3, 17).

   It is highly unlikely that the *N.furzeri* will be able to escape the facility. The facility is a closed system with no release into drainage systems other than that which is done through the water treatment system. All liquid waste that stems from the facility is bleached and/or heat treated prior to drainage.

f. Behaviors that cause environmental degradation

- Behavioural characteristics. Describe any behaviours of the species which cause physical disturbance to the environment e.g. hooves, digging etc.

Whilst egg spawning is associated with very subtle disturbance of the sediment (to help cover the eggs with sediment) (6) no other physical disturbances of the environment have been reported. Additionally, there are Northobranchius species with similar life history traits to that of N. furzeri, already present in Australia, and none of them have had any detrimental effects on our wildlife or environment, which strongly supports our assessment.

- Does the species eat or disturb wetlands/wetland vegetation? This question seeks to identify negative impacts the species may have on wetlands.

  None reported in the many *N. furzeri* habitats studied to date (10, 12).

- Could the species cause pollution of water bodies? This question seeks to identify if the species could impact native aquatic flora or fauna by polluting waterways.

  No. Pollution has not been reported in the many *N. furzeri* habitats studied to date (10, 12).

- If possible, outline the current health of the possible habitat matches in Australia and analyse their sensitivity to possible introductions from the species being assessed.

  There are no particularly suited habitats in Australia and since general impact of N.furzeri on its native environment is low to negligible we predict no deleterious impact of this species on Australian habitats. Additionally, there are Northobranchius species with similar life history traits to that of N. furzeri, already present in Australia, and none of them have had any detrimental effects on our wildlife or environment, which strongly supports our assessment.

g. Impacts on primary industries

- Has the species ever been recorded causing damage to: livestock, poultry, agriculture?

  No, not around the many *N. furzeri* habitats studied (10, 12).

- Could a wild population of the species eat or damage any of the following: plant parts or products; flowers or buds; nuts; root vegetables; leaf vegetables; sugarcane; fodder crops; cotton; nursery/garden plants; timber forests or plantation trees; fruit orchards; stored grain or seeds; legumes; cereal grain in field; oilseeds or coarse grains in field; other (specify).

  No. *N. furzeri* is a generalist predator and feeds on a variety of small aquatic invertebrates with a preference for small crustaceans (Cladocera, Copepoda, Ostracoda and Conchostraca) (16). *N. furzeri* does not eat plants, and there is no reports of damage to plants in the *N. furzeri* habitats studied (10, 12).

- Could wild populations of the species use any resources that might cause it to compete with livestock? This question seeks to identify if this species could compete with livestock.

  No. *N. furzeri* are fish species that do not use any resources that might cause it to compete with livestock (6).

- Has the species ever inflicted damage to trees, shrubs or their seedlings that has caused tree death or affected their value as timber? This question aims to identify if the species may have a negative impact on tree plantations/silvicultural activities.

  No. None of these activities have been reported in habitats inhabited by *N. furzeri* (10, 12).

h. Damage to property

- Could the species deface or physically damage buildings? Identify if the species could damage buildings either through physical damage, or through depositing excrement on the exterior of the building.

  No. Damaged buildings is not evident in habitats inhabited by *N. furzeri* (10, 12), suggesting that this species does not physically damage buildings.

- Could the species damage fences? Identify if the species has the capacity to damage fences.

  No. Damaged fences are not reported in habitats inhabited by *N. furzeri* (10, 12).

- Could the species damage equipment? Identify if the species could cause damage to domestic or commercial equipment.

  No. No damage to equipment has been recorded by *N. furzeri* in natural habitats (10, 12) or in captivity (3).

i. Is the species a social nuisance or danger? For example because of the following behaviours: invading buildings; forming large noisy colonies or flocks; polluting equipment, buildings, parks or other public facilities with urine, droppings or nesting material; posing a risk to aircraft when present in flightways or at airports; other (please specify).

No. None of the behaviors (6) listed above are reported for *N. furzeri* and as such are not considered a social nuisance.

j. Describe any potentially harmful characteristics of the species.

- Any potential threat to humans, any available mitigation measures (such as anti-venom), and methods for appropriate handling.

N. furzeri is a small fresh water species unable to harm humans. *N. furzeri* are not a venomous species (9, 14)and possess no threatening behavioral traits (6). As such, there are no special handing requirements for this species.

- Has the species ever injured people? Identify whether there are any recorded instances of this species causing harm to people.

  No. Wild (1, 10, 12) and captive (3) strains of this species have been handled by humans in several studies and have never been reported to injure humans.

- Is the species susceptible to, or could it transmit any pests or diseases? Identify if the species could potentially transmit harmful diseases or parasites to humans or any other species.

  N. furzeri are susceptible to parasitic infections (detailed in answer to 8(b)), which is similar to all freshwater fish species (3). No particular parasites or diseases apart from disease found in other freshwater species have been reported in N.furzeri.

9. Conditions or restrictions could be applied to the import of the species to reduce any potential negative environmental impacts

As per Australian Department of Agriculture and Water Resources conditions for live laboratory zebrafish imports, live *N. furzeri* (or their eggs) will be imported only from permitted countries of origin as specified by the DAWR. The fish (or eggs) will be inspected by biosecurity officers upon entry and then directed to an aquatic facility at Monash University built to PC2 AS/NZ standards and certified as an OGTR PC2 aquatic facility and DAWR Class 7.2 Approved Arrangement.

During transport, the fish (or eggs) will not be in contact with any other animals which are not in the same consignment and will be shipped in secure, escape-proof containers which are clearly labelled "Live animals under biosecurity control". Container requirements specified by IATA will be met.

Certification will be provided with each consignment to specify the health status of the animals/eggs in the consignment.

Import records for each consignment of fish (or eggs) will be kept by the Approved Arrangement site managers and audited on an annual basis by the DAWR.

Imported fish (or eggs) will be used only for research purposes (detailed in Section 10 below) within the nominated Approved Arrangement facility. Fish and eggs will not be moved from the AA site unless written approval from the DAWR is obtained prior to transfer.

The eggs will be shipped in their dormant state and will only hatch under specific stimuli, which is exposure to cold water and high levels of oxygen saturation that is needed to fill their swim bladder. These requirements will reduce their impact on the environment.

Additionally, in line with protocols established for *N. furzeri* (3) and zebrafish (18), *N. furzeri* eggs will be washed in sodium hypochlorite (household bleach) prior to importation , which will sterilize them hence minimizing the risk of pathogen transmission and the impact on the environment.

10. Summary of proposed activity

- The rationale for choosing to import this species into Australia.

  We wish to import *N. furzeri* for research on ageing biology.

  Ageing is an inevitable, multisystemic disorder characterized by a progressive decline in the ability of organs to perform their physiological functions. *N. furzeri* has the shortest known maximum lifespan of a vertebrate species that can be bred in captivity making it an ideal model for the study of ageing biology, and an optimal platform to develop therapies for ageing and age dependent diseases such as cancer, obesity and diabetes (7, 19-22). *N. furzeri* is a very important research tool for research on ageing and has the potential to reveal the many unresolved questions pertaining to this inevitable condition.

- Clearly state the numbers of animals you want to import.

  Approximately 1000 eggs and 100 adults (50 males and 50 females).

- Discuss the interaction between males of this species. Do they need to be segregated?

  In general, groups of *N. furzeri* coexist peacefully, but during the week or two during which they mature there may be some fighting as an invisible hierarchy is established. Male fish are best sorted into groups based on size (3, 6, 11).

- If the purpose is for breeding discuss the management and control of excess progeny in the breeding program. How many animals will be kept at any time on the premises? How will lack of genetic variation be managed in the breeding program?

  For efficient and successful research we will maintain a maximum of 50 mating pairs of *N. furzeri* at a time. Excess progeny will be maintained as dormant eggs, which will act as a stock reserve for future breeding pairs.

  The lack of genetic variation is not a problem for maintaining *N. furzeri*. We hope to import the laboratory strain (GRZ strain) of *N. furzeri* which has undergone approximately 80 captive generations , suggesting that the high levels of inbreeding and lack of genetic variation is not an issue (1, 20).

- Discuss any other potential uses for this species should it be imported into Australia. Where applicable, describe its human uses (e.g. zoos, research, pets etc.).

  N. furzeri have the shortest known lifespan of a vertebrate species that can be bred in captivity (19). This makes it an ideal animal model for research on ageing and age-related diseases, which is what the species will be used for (7, 23, 24). Additionally, *N. furzeri* will be used for science education and student outreach programs.

- Provide details on where animals are obtained, e.g. captive bred populations or from the wild.

  Captive bred populations will be obtained from overseas research facilities

11. Guidelines on how species should be kept

- What are the standards for transporting animals? Will the animals be transported according to International Air Transport Association (IATA) regulations?

  As per DAWR Biosecurity requirements, animals will be shipped in secure, escape-proof containers which are clearly labelled "Live animals under biosecurity control". Container requirements specified under IATA will be met.

  Transport containers and all material secured therein (excluding the live fish) will be disposed of by incineration or disinfection (by autoclaving or treatment with chlorine (200 ppm final concentration) or Virkon prior to disposal).

  Imported water, wastewater used in the approved arrangement site used to house the fish and all water contacting the live fish will be treated prior to release from the AA site in accordance with the AA site requirements and the approved operating procedures.

- How does enclosure size relate to territory requirements?

  Given that *N. furzeri* is not an established species in Victoria, there are currently no territory requirements established for this species. The enclosures used to house the species at the facility in Monash University will be 10L tanks with recirculating water.

- Discuss the containment and management standards for Australia e.g. the proportion of males to females and the maximum number that should be kept in enclosures/aquaria. Also if single sex populations would be contained within enclosures to limit breeding etc.

  Protocols have been published suggesting the density at which the species should be maintained (3, 25): for the first 4 days after hatching 200-300 juvenile can be maintained in 6L of water; from 4 days to 4 weeks of age, it is recommended that *N. furzeri* are kept at a density of 1-2L per fish; and adults above the age of 4 weeks at a density of 10-15 fish per 30L. These recommendations are based on systems

without water recirculation where the density of fish is limited due to the build of metabolic waste products.

We will use professional zebrafish rack systems, which have been shown to be successful for housing *N. furzeri* (3). These systems use recirculating water and as such the density at which the species is housed can be increased. We will maintain 100-150 juvenile below that age of 4 days in 3L tanks, and fish above the age of 4 days will be maintained at a density of 10-15 fish, with equal male to female ratios, fish in 10L tanks. Aggression of the dominant male is greatly reduced if fishes are kept in large groups. To avoid excessive harassment of female fish we will maintain equal or female biased sex ratios (3). Whilst single sex populations can be maintained it is advisable not to as female fish need to spawn ovulated eggs to retain reproductive capacity, and the failure to lay eggs may be fatal for them.

- What standards are used for the enclosures/aquaria in which this species would be kept? What are the best practice standards? Who applies these standards? Will enclosures/aquaria be sufficiently large enough for the humane containment of the animals? For example providing sufficient depth and length?

  Species will be housed in a site certified as an OGTR PC2 aquatic facility and Department of Agriculture and Water Resources Class 7.2 Approved Arrangement.

  Additionally, two detailed protocols published by highly experienced researchers in maintaining *N. furzeri* have been published (3, 25). The protocols clearly outline the best practice for maintaining and breeding *N. furzeri*, including feeding regimes, water management guidelines, enclosure specs, quarantine procedures, treatment plans and troubleshooting guides. Ethics has been obtained to maintain, breed and perform research on *N. furzeri* from the Monash animal ethics committee. The researchers and aquarium staff will follow the ethics guidelines to ensure the humane treatment of the species.

- Address welfare issues in housing captive specimens.

  Welfare issues including housing, diet, cleanliness, water quality, health related issues have been addressed in the protocols included in (3, 25).

12. State/Territory controls

Two killifish species, *Aphyosemion spp.* and *Epiplatys spp.*, are in Part 1 of the List of Specimens Suitable for Live Import and so are approved for import into Australia without a permit from the federal environment department.

Nothobranchius furzeri is NOT a CITES species.

An import permit will be obtained from the Department of Agriculture and Water Resources - Biosecurity division before any import of *N. furzeri* will occur. Imported *N. furzeri* will be housed in a site certified as an OGTR PC2 aquatic facility and Department of Agriculture and Water Resources Class 7.2 Approved Arrangement.

As the *N. furzeri* will not be released from the AA site, there will be no impact on the Victorian environment. The Victorian Wildlife Act 1975 and Wildlife Regulations 2013 do not apply to *N. furzeri* as it is not a native species.

References

1. E. Terzibasi *et al.*, Large differences in aging phenotype between strains of the short-lived annual fish Nothobranchius furzeri. *PLoS ONE*. **3**, e3866 (2008).

2. R. A. Jubb, A New Nothobranchius (Pisces, Cyprinodontidaej From Southeastern Rhodesia, 1–4 (1971).

3. M. Polačik, R. Blažek, M. Reichard, Laboratory breeding of the short-lived annual killifish Nothobranchius furzeri. *Nat Protoc*. **11**, 1396–1413 (2016).

4. J. Kirschner *et al.*, Mapping of quantitative trait loci controlling lifespan in the short-lived fish Nothobranchius furzeri--a new vertebrate model for age research. *Aging Cell*. **11**, 252–261 (2012).

5. M. Polačik, M. T. Donner, M. Reichard, Age structure of annual Nothobranchius fishes in Mozambique: is there a hatching synchrony? *J. Fish Biol.* **78**, 796–809 (2011).

6. A. Cellerino, D. R. Valenzano, M. Reichard, *Biol Rev Camb Philos Soc*, in press, doi:10.1111/brv.12183.

7. T. Genade *et al.*, Annual fishes of the genus Nothobranchius as a model system for aging research. *Aging Cell*. **4**, 223–233 (2005).

8. B. R. Watters, The ecology and distribution of Nothobranchius fishes. **42**, 37–76.

9. B. Nicolas, Nothobranchius furzeri summary page. (available at http://fishbase.org/summary/9855).

10. M. Reichard, M. Polačik, O. Sedlácek, Distribution, colour polymorphism and habitat use of the African killifish Nothobranchius furzeri, the vertebrate with the shortest life span. *J. Fish Biol.* **74**, 198–212 (2009).

11. M. Polačik, M. Reichard, Asymmetric reproductive isolation between two sympatric annual killifish with extremely short lifespans. *PLoS ONE*. **6**, e22684 (2011).

12. M. Reichard, M. Janáč, M. Polačik, R. Blažek, M. Vrtílek, Community assembly in Nothobranchius annual fishes: Nested patterns, environmental niche and biogeographic history. *Ecol Evol*. **7**, 2294–2306 (2017).

13. R. Haas, Behavioral Biology of the Annual Killifish, Nothobranchius guentheri. *American Society of Ichthyologists and Herpetologists*. **1976**, 80–91 (1976).

14. *NFIN - The Nothobranchius furzeri Information Network*. (available at w*ww.nothobranchius.infopagesbiologylifecycleecologydiet.php.* (2011).

15. M. Bomford, J. Glover, "*Risk assessment model for the import and keeping of exotic freshwater and estuarine finfish*" (2004).

16. M. Polačik, M. Reichard, Diet overlap among three sympatric African annual killifish species Nothobranchius spp. from Mozambique. *J. Fish Biol.* **77**, 754–768 (2010).

17. D. R. Valenzano, E. Terzibasi, A. Cattaneo, L. Domenici, A. Cellerino, Temperature affects longevity and age-related locomotor and cognitive decay in the short-lived fish Nothobranchius furzeri. *Aging Cell*. **5**, 275–278 (2006).

18. M. Westerfield, *The Zebrafish Book; A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio)* (University of Oregon Press, Eugene, ed. 2, 1993).

19. S. Valdesalici, A. Cellerino, Extremely short lifespan in the annual fish Nothobranchius furzeri. *Proc. Biol. Sci.* **270 Suppl 2**, S189–91 (2003).

20. E. Terzibasi, D. R. Valenzano, A. Cellerino, The short-lived fish Nothobranchius furzeri as a new model system for aging studies. *Exp. Gerontol.* **42**, 81–89 (2007).

21. C.-Y. Hsu, Y.-C. Chiu, W.-L. Hsu, Y.-P. Chan, Age-related markers assayed at different developmental stages of the annual fish Nothobranchius rachovii. *J. Gerontol. A Biol. Sci. Med. Sci.* **63**, 1267–1276 (2008).

22. R. Blažek, M. Polačik, M. Reichard, Rapid growth, early maturation and short generation time in African annual fishes. *Evodevo*. **4**, 24 (2013).

23. D. R. Valenzano, A. Cellerino, Resveratrol and the pharmacology of aging: a new vertebrate model to validate an old molecule. *Cell Cycle*. **5**, 1027–1032 (2006).

24. S. Wendler, N. Hartmann, B. Hoppe, C. Englert, Age-dependent decline in fin regenerative capacity in the short-lived fish Nothobranchius furzeri. *Aging Cell*. **14**, 857–866 (2015).

25. T. Genade, Laboratory manual for culturing *N. furzeri* (2005).