The biology of aging in *Nothobranchius* fishes

Biologie stárnutí anuálních ryb rodu *Nothobranchius*

Jakub Žák

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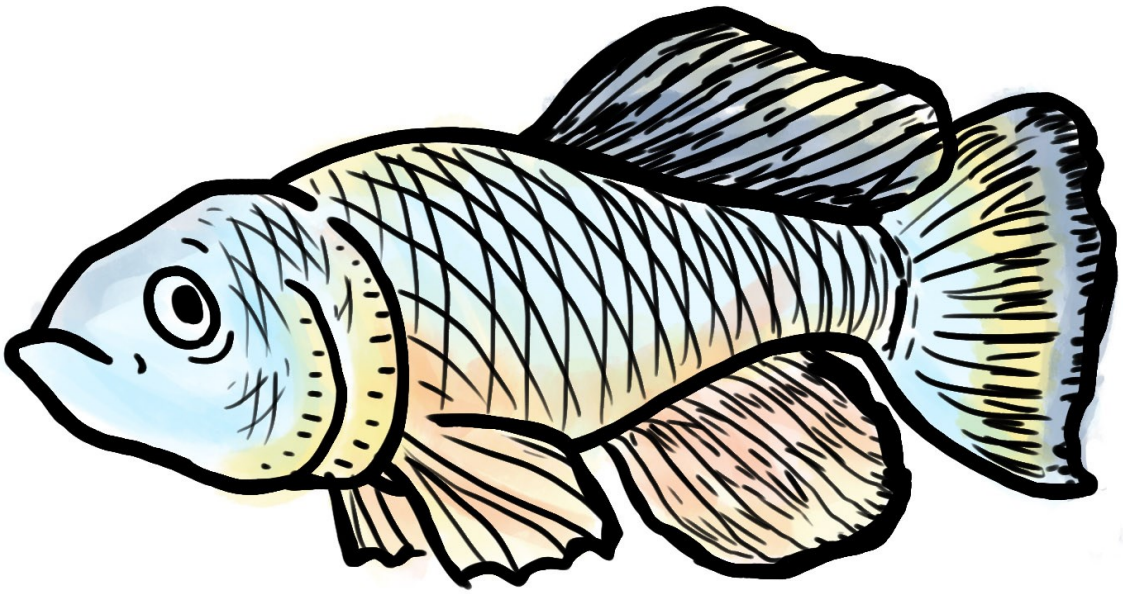
Biologie stárnutí anuálních ryb rodu *Nothobranchius*

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PROHLÁŠENÍ

Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu. Tuto práci jsem zpracoval samostatně a uvedl všechny použité informační zdroje a literaturu.

V Brně dne

Jakub Žák

DECLARATION

I declare that this dissertation or its significant part was not submitted for the purpose of obtaining the same or any other academic degree. All literature sources I used when writing this thesis have been properly cited.

Brno, Czech Republic

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PAPER 1

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PAPER 3

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Vrtílek, M., **Žák, J**., Polačik, M., Blažek, R., & Reichard, M. (2018). Longitudinal demographic study of wild populations of African annual killifish. Scientific Reports, 8(1), 4774

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PAPER 5

Vrtílek, M., **Žák, J**., Blažek, R., Polačik, M., Cellerino, A., & Reichard, M. (2018). Limited scope for reproductive senescence in wild populations of a short-lived fish. The Science of Nature, 105(68).

Citations (Google Scholar|Web of Knowledge) = 16|13; IF = 1.84; JIFQ = Q2

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prof. RNDr. Martin Reichard, PhD.

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Žák, J., & Reichard, M. (2021). Reproductive senescence in a short-lived fish. Journal of Animal Ecology, 90(2), 492-502.

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PAPER 7

Žák, J., Vrtílek, M., & Reichard, M. (2019). Diel schedules of locomotor, reproductive and feeding activity in wild populations of African annual killifish. Biological Journal of the Linnean Society, 128(2), 435-450.

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PAPER 8

Žák, J., Reichard, M., & Gvoždík, L. (2018). Limited differentiation of fundamental thermal niches within the killifish assemblage from shallow temporary waters. Journal of Thermal Biology, 78, 257-262.

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Dyková, I., **Žák,** J., Reichard, M., Součková, K., Slabý, O., Bystrý, V., & Blažek, R. (2021). Histopathology of laboratory-reared *Nothobranchius* fishes: Mycobacterial infections versus neoplastic lesions. Journal of Fish Diseases (44)8, 1179-1190.

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ABSTRACT

The aging human population and consequent unprecedented demographic changes in society have inevitably led to more scientists focusing their research on aging. Senescence, in the narrowest biological sense, is age related decline in an organism's function and survival. The majority of senescence research is performed in the laboratory environment. The basic biology of wild counterparts of model organisms is therefore relatively unstudied, despite its importance for proper interpretation of laboratory outcomes. This dissertation, via a series of 6 first-authored and 5 second-authored papers, combines field and laboratory research on senescence and senescence-related life history traits using a short-lived fish *Nothobranchius furzeri*. An age at the maturity of 14 days post hatching in wild *N. furzeri* is presented in this thesis as well as extremely fast growth of wild fish. Both life history traits have important consequences for senescence. The following section is focused on how the environment modulates senescence. In the first step, preferred body temperatures of Mozambican *Nothobranchius* fish were measured and descriptions of environmental and behavioural fluctuations within a 24 hours periodicity were completed. Based on this knowledge, ecologically relevant thermal fluctuations were simulated in captivity to test how this factor affects senescence. Fish experiencing fluctuations had superior survival and relative reproduction. This finding questions the precision of experimental results obtained under constant temperature. Another set of experiments compared the appearance of reproductive senescence in wild fish and in captivity. Wild fish do not live long enough to develop reproductive senescence while captive fish experienced reproductive senescence at advanced age. This dissertation clearly demonstrates how important accurate knowledge of fundamental species biology is for proper design of experiments as well as how important findings from wild populations are for interpretations of laboratory research.

ABSTRAKT

Stárnutí lidské populace a následné bezprecedentní demografické změny ve společnosti vedly nevyhnutelně ke zvýšené pozornosti vědců k výzkumu stárnutí. Stárnutí je v biologii definováno jako pokles kvality fyziologických funkcí a rostoucí riziko úmrtí související s rostoucím věkem. Většina výzkumu stárnutí se provádí v laboratorním prostředí. Proto existuje velmi málo poznatků o biologii volně žijících populací u druhů, které jsou využívány jako modelové organismy v laboratoři. Nicméně jejich znalost je důležitá pro správnou interpretaci laboratorních výsledků. Tato disertační práce prostřednictvím šesti prvoautorských a pěti druhoautorských prací kombinuje poznatky z terénního a laboratorního výzkumu stárnutí (a životních strategií souvisejících se stárnutím) krátkověkého halančíka *Nothobranchius furzeri*. V práci je popsán rychlý vývin halančíka, který je schopen v přírodě dospět během 14 dní od vylíhnutí a také jeho extrémně rychlý růst v přírodě. Obě tyto životní strategie zásadně ovlivňují stárnutí organismu. Další část dizertace popisuje, jak prostředí ovlivňuje stárnutí. Nejprve byly zjištěny preferované tělesné teploty halančíků rodu *Nothobranchius,* charakterizovány denní výkyvy prostředí a chování v divokých populacích. Na základě těchto poznatků byl testován vliv ekologicky relevantních teplotních fluktuací na stárnutí. Halančíci z prostředí s cirkadiánní teplotní fluktuací lépe přežívali a měli vyšší relativní plodnost než halančíci chovaní ve stabilní teplotě. Toto zásadní zjištění může zpochybnit přesnost experimentálních výsledků získaných během chovu za stabilních teplot. Další experimenty porovnaly reprodukční stárnutí u volně žijících halančíků a u halančíků v zajetí. Bylo zjištěno, že volně žijící halančíci nežijí dostatečně dlouho na to, aby se u nich projevilo reprodukční stárnutí. Naopak u halančíků chovaných v zajetí se reprodukční stárnutí projevilo v pokročilém věku. Tato disertační práce jasně ukazuje, jak důležitá je znalost základní biologie druhu pro správné naplánování experimentů a jak důležité jsou poznatky získané studiem volně žijících populací pro interpretaci laboratorního výzkumu.

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INTRODUCTION

Humankind has never experienced such a long lifespan as we do today. This revolution in longevity is the outcome of the industrial era in the 18th century and consequent advances in science, healthcare, sanitation and wellbeing. Since then, society has begun to experience unprecedented medical and socially adverse consequences such as diabetes, cancer, dementia and increased demand for care of the elderly (Vaupel, 2010). When these challenges are combined with a decline in birth-rate of wealthy countries, some scientists warn against an epidemic of ageing (Newman & Cauley, 2012). Thus, the increased attention of scientists on understanding the aging process and how to intervene is inevitable.

Aging has various definitions depending on the field of study and related organization (i.e. individual, population). Aging may mean just an increase in age as time passes, without apparent negative outcomes for the organism (Shefferson et al., 2017a) as well as physiological decline with chronological age (Reichard, 2020). Senescence is aging in the narrowest biological sense, when an organism suffers from age-related physiological decline expressed as reduced reproduction, immunity and/or survival (Gaillard & Lemaître, 2017; Froy et al., 2019). Aging and senescence may be used interchangeably (Shefferson et al., 2017b) but the context should always be well explained. Here, in this text accompanying the published works, the term senescence is used to mean a decline in organismal function or survival. However, the terminology will differ among publications which is the outcome of different scope and requirements of specific journals, editors and peer-reviewers.

Senescence is a complex problem which requires a multidisciplinary approach with synergy of various scientific disciplines and sub-disciplines such as evolutionary biology, ecology, demography, zoology, experimental biology, biomedicine and others. Model organisms are a core tool used for answering enigmatic problems in biology, including senescence (Ram & Conn, 2018). They help scientists to think in innovative ways and find

new solutions to problems. Newly emerging questions in biology often benefit from new model organisms with particular traits to answer specific questions (Bolker, 2012; Alfred & Baldwin, 2015). The vast majority of senescence research is performed on a short list of models such as the fruit fly *Drosophila melanogaster*, roundworm *Caenorhabditis elegans* or mice *Mus musculus* (Reznick et al., 2002; Gerhard et al., 2004). However, there is debate on how representative these organisms are (Bolker, 2012; Alfred & Baldwin, 2015), thus there is growing attention on other model species such as short-lived fishes (Ackerman & Gerhard, 2018; Hu & Brunet, 2018). Some short-lived fishes exhibit shortest lifespans among vertebrates (Valdesalici & Cellerino, 2003). Currently, one of the most promising fish models for senescence research is *Nothobranchius furzeri* (Astre et al., 2022), a small fish from African savanna temporary pools (Reichard & Polačik, 2019). *Nothobranchius furzeri* was introduced into biogerontological research nearly twenty years ago (Valdesalici & Cellerino, 2003; Genade et al., 2005). This dissertation builds on the utility of this species and investigates senescence and senescence-related life history traits of *Nothobranchius furzeri* using a combination of field observations and laboratory experiments.

EVOLUTIONARY THEORIES OF SENESCENCE

At first glimpse, senescence itself may seem contradictory when considered from the perspective of evolutionary theory. Why do organisms lose their ability to maintain an existing complex body (Williams, 1957; Kirkwood, 1977)? Why does evolution allow the decline of fitness at advanced age when the "survival of the fittest" is considered central dogma of evolutionary theory (Darwin, 1859)? The "Modern Synthesis" of evolutionary biology (Huxley, 1942) which interlinked evolutionary processes with genotype allowed us to better understand the complexity of senescence. It provided us with (at least partial) answers to why senescence occurs across the tree of life as well as why some organisms suffer more from senescence (Medawar, 1952; Williams, 1957; Hamilton, 1966; Kirkwood, 1977).

Nonetheless, senescence is still far from resolved within the evolutionary paradigm (Jones & Vaupel, 2017; Reichard, 2017; Cohen et al., 2020).

The history of evolutionary theories of senescence dates back at least to the 19th century when August Weismann introduced the "concept of programmed death" (Weismann, 1891). In Wiesmann's theory, senescence is evolutionary maintained because younger and more fecund individuals benefit from the death of old individuals due to consequent reduction of competition within the population. This reduction contributes to the increase of species-specific fitness. One major caveat of Weismann's concept is that it neglects the role of extrinsic mortality and thus the theory does not explain why organisms from harsh environments have shorter lifespans than organisms from protected conditions (Medawar, 1952; Ljubuncic & Reznick, 2009).

In the middle of the 20th century, Peter Medawar proposed the "Mutation accumulation theory of senescence" (Medawar, 1952). Medawar's theory proposed that natural selection at increased age weakens because older individuals continuously die-off (due to senescence and extrinsic mortality) in a population and thus contribute only little to the pool of offspring. Their reduced reproductive capacity causes the decline in strength of natural selection at later age (called "selection shadow"). Selection shadow enables the persistence of late-acting negative mutations in the population, because they were already passed on to the next generation prior to their negative consequences being expressed. In contrast to Weismann's theory, Medawar's theory explains the shorter lifespan of organisms from high mortality environments versus protected low mortality environments (see e.g. Austad, 1993). In high mortality environments organisms accumulate more negative mutations and consequently evolve an intrinsically shorter lifespan.

The presence of selective shadow is a prerequisite also for another popular theory – "Antagonistic pleiotropy" which was first proposed by George C. Williams (Williams, 1957).

Antagonistic pleiotropy builds on the theory of late-acting negative mutations but considers the negative effects as a by-product of positively selected pleiotropic alleles acting early in life. This theory therefore links increased fitness in early life with earlier onset of senescence.

The "Disposable soma" theory (Kirkwood, 1977) is based on the prerequisite of somatic and germ line separation suggested already by Weismann. Disposable soma theory assumes that there is a trade-off in resource allocation between the somatic and germ line cells. The transfer of genes to successive generations is essential for the evolution of organisms and thus investment into the germ line comes with higher long term benefits than maintenance of the somatic cells (Dawkins, 1976; Kirkwood, 1977). Resources are therefore primarily allocated to the maintenance of germ-line cells at the expense of somatic cells which leads to continuous accumulation of damage in somatic cells which is later express as senescence.

There are plenty of other evolutionary and mechanistic theories of senescence (e.g. the free radical theory: Beckman & Ames, 1998; positive pleiotropy: Maklakov et al., 2015; inflamm-aging: Franceschi et al., 2000; tumor suppression theory: Wolf, 2021; hyperfunction theory: Blagosklonny, 2008). Despite an abundance of theories and the increasing attention of researchers towards the field of senescence, we currently principally describe the symptoms of senescence instead of investigating the evolution of their origin (Vleck et al., 2007; López-Otín et al., 2013; Reichard, 2017). This contributes to the lack of consensus on the universal theory of senescence (Cohen et al., 2020). Nonetheless, it confirms that studying the evolutionary roots of senescence is extremely important not only for developing better interventions (biomedical research) but also for proper understanding of the world around us.

SENESCENCE IN ORGANISMS WITH INDETERMINATE GROWTH

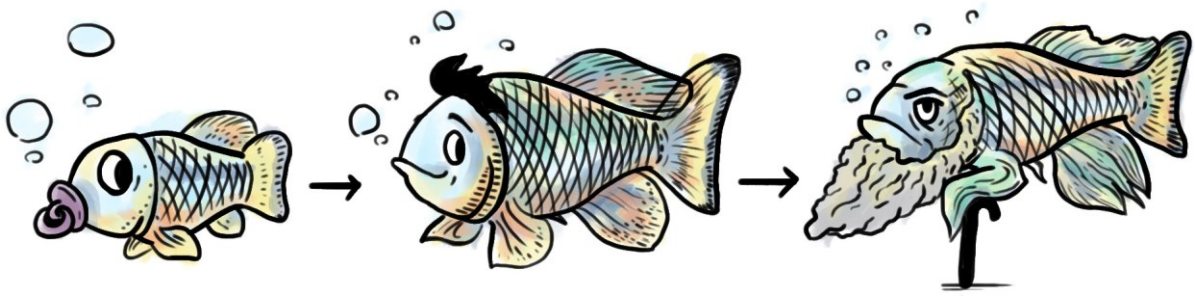
There is huge diversity in senescence across multicellular organisms (Jones et al., 2014; Jones & Vaupel, 2017). Organisms with indeterminate growth are predicted to

completely avoid senescence or experience only “negligible senescence” (Vaupel et al., 2004; Jones & Vaupel, 2017). This is based on the assumption that the fitness (e.g. fecundity, competitive ability, survival) of these organisms is closely positively related to their body size and thus natural selection would favour large (and consequently old) individuals over small ones (Vaupel et al., 2004). Potential escape from senescence in these organisms with indeterminate growth makes them exciting models for investigating the senescence process and how to intervene. On the other hand, the assumption of negligible senescence in organisms with post-maturity growth may simply be an artefact caused by data deficiency (Warner et al., 2016).

Senescence is mostly studied in model organisms with cessation of growth at maturity such as the fruit fly, roundworms or mice (Reznick et al., 2002; Gerhard et al., 2004). In addition, the theoretical substrate for senescence is best developed for determinate growers (Reznick et al., 2002; Jones & Vaupel, 2017). The use of a relatively short list of organisms with determinate growth thus provides only limited insights into the diversity of senescence patterns and considerable knowledge gaps remain in the senescence process of organisms with indeterminate growth (Reznick et al., 2002). More thorough investigation of organisms with indeterminate growth will broaden knowledge about senescence diversity.

Fishes (Teleostei/Actinopterygii) represent a particularly rich and diverse group of vertebrates with indeterminate growth (Reznick et al., 2002). There is, however, only limited knowledge about whether they do, or do not, suffer from senescence (Patnaik et al., 1994; Reznick et al., 2002). Fishes are popular models in many biological disciplines (D’Abramo & de Girolamo, 2022) and they have great potential to be useful models for investigating the evolutionary roots of senescence in vertebrates (Cellerino et al., 2016; Ackerman & Gerhard, 2018). Their utility is supported by growing evidence that the senescent phenotype is shared among the majority of vertebrates (Cellerino et al., 2016; Reichard, 2016a). Recently, an

extremely short-lived killifish *Nothobranchius furzeri* was introduced as a promising model for senescence research (Genade et al., 2005; Cellerino et al., 2016; Astre et al., 2022).



LIFE HISTORY TRAITS OF SHORT-LIVED KILLIFISH FROM GENUS *NOTHOBRANCHIUS*

Annual killifish from the genus *Nothobranchius* (Class: Actinopterygii, family: Nothobranchiidae) are known for their exceptionally rapid life history and short lifespan, therefore, they have become a promising model for senescence-oriented research (Genade et al., 2005; Cellerino et al., 2016; Hu & Brunet, 2018). *Nothobranchius furzeri* is especially gaining in popularity because it has the shortest lifespan of any vertebrate that can be successfully bred in captivity (Valdesalici & Cellerino, 2003). Its captive lifespan is counted in weeks or months rather than years (Cellerino et al., 2016). *Nothobranchius furzeri* originates from shallow periodically desiccating savanna pools in south-east Africa (Reichard et al., 2009). Adaptations to this environment entail desiccation-resistant embryos that enter three metabolism-reducing diapause stages, fast maturity, fast growth and high fecundity (reviewed in Reichard & Polačik, 2019). Currently, there is a variety of wild-derived and also inbred laboratory strains of *N. furzeri* (Cellerino et al., 2016; Reichard et al., 2022). *Nothobranchius furzeri* is a genetically tractable model with a sequenced and annotated genome (Reichwald et al., 2015; Valenzano et al., 2015; Willemsen et al., 2020) and established gene-editing protocol (Harel et al., 2016). A more detailed description of their biology and laboratory application can be found in various reviews (Genade et al., 2005;

Lucas-Sánchez et al., 2014; Cellerino et al., 2016; D'Angelo, 2017; Hu & Brunet, 2018; Philippe et al., 2018; Reichard & Polačik, 2019; Tozzini & Cellerino, 2020).

The fast pace of life combined with a long period of post-maturity growth of *N. furzeri* (Blažek et al., 2013) provides a unique opportunity to test conflicting evolutionary theories of senescence. As mentioned above, organisms with indeterminate growth are expected to experience only negligible senescence (Vaupel et al., 2004). On the other hand, a rapid lifecycle is expected to come with the cost of early and fast senescence (Jones et al., 2008). A recent evolutionary genomic investigation found that mutation accumulation appears to be primarily responsible for the short lifespan of *N. furzeri* (Willemsen et al., 2020). However, how such an accumulation of mutations in *N. furzeri* is expressed in the senescence phenotype is still being studied and is the core of this dissertation.

AIMS OF THE DISSERTATION PROJECT

My dissertation project is aimed at investigating senescence and senescence-related life-history traits of *Nothobranchius furzeri* using a multidisciplinary approach. The aims can be generally divided into five points:

1/ to provide insights into the wider evolutionary-ecological context of fish species (especially outside African annual killifish) with a natural lifespan shorter than one year;

2/ to identify essential ecological and life history traits which affect senescence or are markers of senescence from *N. furzeri* in the wild (diurnal activity, growth rate, age at maturity) and in a laboratory (thermal requirements)

3/ to describe the demography of *N. furzeri* in their natural habitat including senescence in survival and sex-specific mortality

4/ to test how the environment affects senescence of *Nothobranchius furzeri*

(a) reproductive senescence of wild fish in their natural habitat vs captive populations under laboratory conditions

(b) stable temperature conditions vs experimentally induced and ecologically relevant thermal fluctuations in the laboratory

5/ to address outstanding challenges of *Nothobranchius* laboratory husbandry and health indicators

a) establish a replacement for the currently used live food with a pelleted diet

b) review histopathology of *Nothobranchius* in laboratory colonies

All these aspects were studied using a combination of field and laboratory research. New findings on life histories in the wild (reproductive senescence in wild populations, daily fluctuations in natural environment and fish behaviour) and in captivity (thermal requirements) were then investigated in laboratory experiments (captive reproductive senescence; impact of temperature fluctuation on *N. furzeri* senescence). During these laboratory studies, several practical aspects that required further elaboration were identified. These were related to the replacement of live food which is currently recognized as the dietary "standard" for *Nothobranchius* fish (Polačik et al., 2016) but has several shortcomings for fish husbandry. Another topic was histopathology of *Nothobranchius* fishes where misdiagnoses may have occurred in some previous studies on their senescence. Data for this dissertation were collected as part of a five-moth fieldwork project in the Mozambican savanna that was followed by 30 months of laboratory experimental work. The above outlined aims are addressed and developed by 6 first-author papers and 5 second-authored papers.

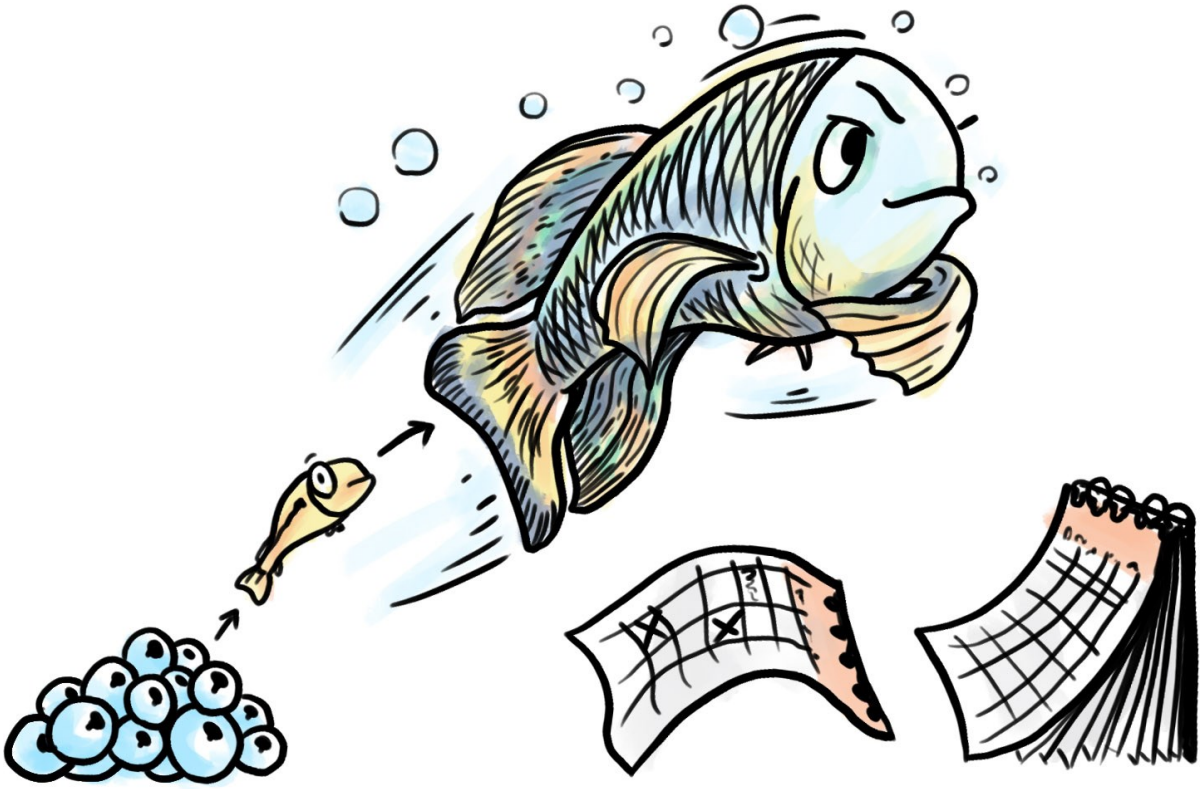
OUTLINE OF THE THESIS AND BRIEF SUMMARY OF KNOWLEDGE GAPS IN BIOLOGY AND HUSBANDRY OF *NOTHOBRACHIUS FURZERI* RELATED TO SENESCENCE

Killifish from the genus *Nothobranchius* are not the only fish species with a lifespan shorter than one year. Knowledge about the occurrence of annual lifespans within ray-finned fish is, however, very limited. This is particularly pressing as short-lived fishes represent promising models for investigation of vertebrate senescence (Ackerman & Gerhard, 2018). This lack of information is especially apparent outside the group of annual killifish (reviewed in Berois et al., 2016). **Paper 1** provides a systematic review of the diversity of short-lived fishes and synthetizes their common ecological and evolutionary patterns. The review also challenges the position of *Eviota sigillata* as a candidate vertebrate with the shortest lifespan. Species from *Schindleria* genus are serious candidates (estimated maximum age <30 days) but a validated method for determining their age does not exist. In addition, wild *Nothobranchius furzeri* is also extremely short-lived because it maintains viable natural populations in pools which desiccate prior to the onset of maturity of *Eviota sigillata*. In addition, this review offers a new definition of multivoltine fish species whose "shortevity" (sensu Ackerman & Gerhard, 2018) enables them to produce multiple generations within a single year.

The crucial principles of senescence are difficult to reveal without proper understanding of the model organisms' essential life history traits (Alfred & Baldwin, 2015; Reichard, 2016a; Reichard & Polačik, 2019). Unfortunately, research resources are mostly devoted to the investigation of the senescence phenomenon instead of studying the essential biology of the model organism (Spence et al., 2008; Alfred & Baldwin, 2015; Reichard & Polačik, 2019). Our knowledge on the natural biology of certain model organisms is still daringly limited. For example, the natural host fruit of the laboratory fly *Drosophila melanogaster* was unknown until relatively recently (Mansourian et al., 2018). As an

emerging model organism, *N. furzeri* also suffers from knowledge gaps in its natural history despite notable recent advances (Reichard, 2016b; Reichard & Polačik, 2019). Some questions pertaining to their husbandry under laboratory conditions still remain.

Theory and empirical data suggest a tight relationship between age at maturity and lifespan (Williams, 1957; Kirkwood, 1977) as well as between lifespan and growth rate (Kirkwood, 1977; Lee et al., 2013). In *N. furzeri,* maturity in captivity was thought to be delayed and growth rate retarded when compared with wild populations (Blažek et al., 2013). No empirical data to make a direct comparison were available though. Intensively fed *N. furzeri* may reach maturity within three weeks post-hatching but it was not clear whether this was an artefact of "optimal" conditions in captivity or a "natural" phenomenon (Blažek et al., 2013). **Paper 2** provides evidence of extremely rapid maturation of *N. furzeri* in their natural pools and clearly demonstrates, based on gonad histology, that wild fish can reach maturity within 14 days post hatching. This is the most rapid sexual maturation in any vertebrate ever

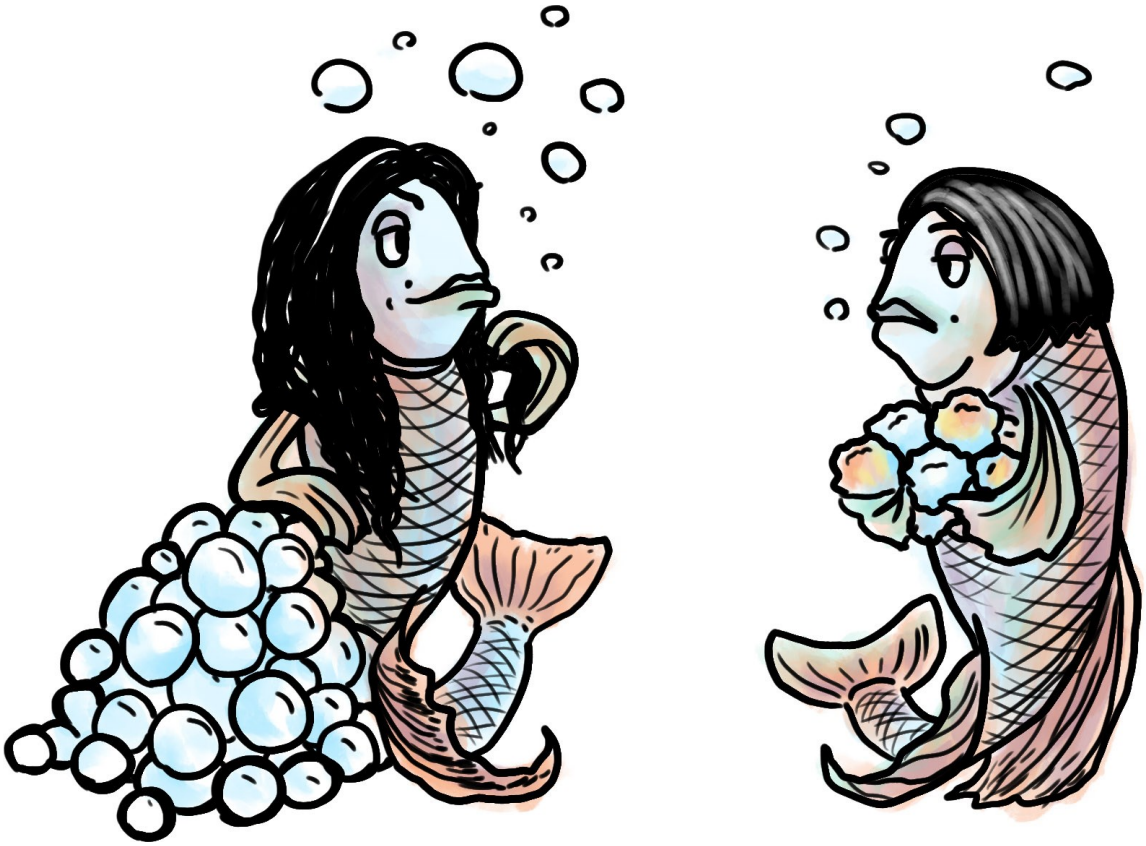


recorded. **Paper 3** estimated growth patterns in wild *Nothobranchius* species in relation to conspecific density in the pool. The extremely quick maturity, exceptional among vertebrates, and the apparent fast growth rate of wild *Nothobranchius* spp. suggests that senescence in captivity and the wild may differ significantly. Thus, interconnection of empirical data on senescence from the wild and the laboratory was necessary.

The natural life history of *Nothobranchius* fish in the wild is relatively poorly known and almost nothing is known about the onset and rate of their senescence in the wild. Describing the expression of senescence in the wild is essential for proper understanding of its evolutionary origin and the environmental conditions that regulate it (Monaghan et al., 2008; Reichard, 2016a). In addition, differences measured in the wild and in captivity may provide important insights into understanding the mechanisms of senescence. Antagonistic pleiotropy predicts the occurrence of senescent individuals both in the wild and captivity (Williams, 1957). In contrast, Mutation accumulation theory expects that senescent individuals will be extremely rare in the wild, while senescence could be seen at advanced age in captive individuals (Medawar, 1952). From a different perspective, the harsh stressful conditions of the natural habitat may increase oxidative damage, for example, and consequently expedite the onset of senescence (Beckman & Ames, 1998; Selman et al., 2012). **Paper 4** provides the first demographic data on age-related survival (actuarial senescence) in wild populations of *N. furzeri*. It was particularly interesting to see that in some pools the decline in fish abundance was much faster than the decrease in pool area. This resulted in lower fish density at later stages of the pool's existence, entirely in contrast to the general expectation of pool desiccation being the main extrinsic selection driver for *Nothobranchius* lifespan. **Paper 5** shows age-related allocation to reproduction in wild *N. furzeri* females and males, a strong decline with increasing age was not found. These findings are critical for articulating follow-

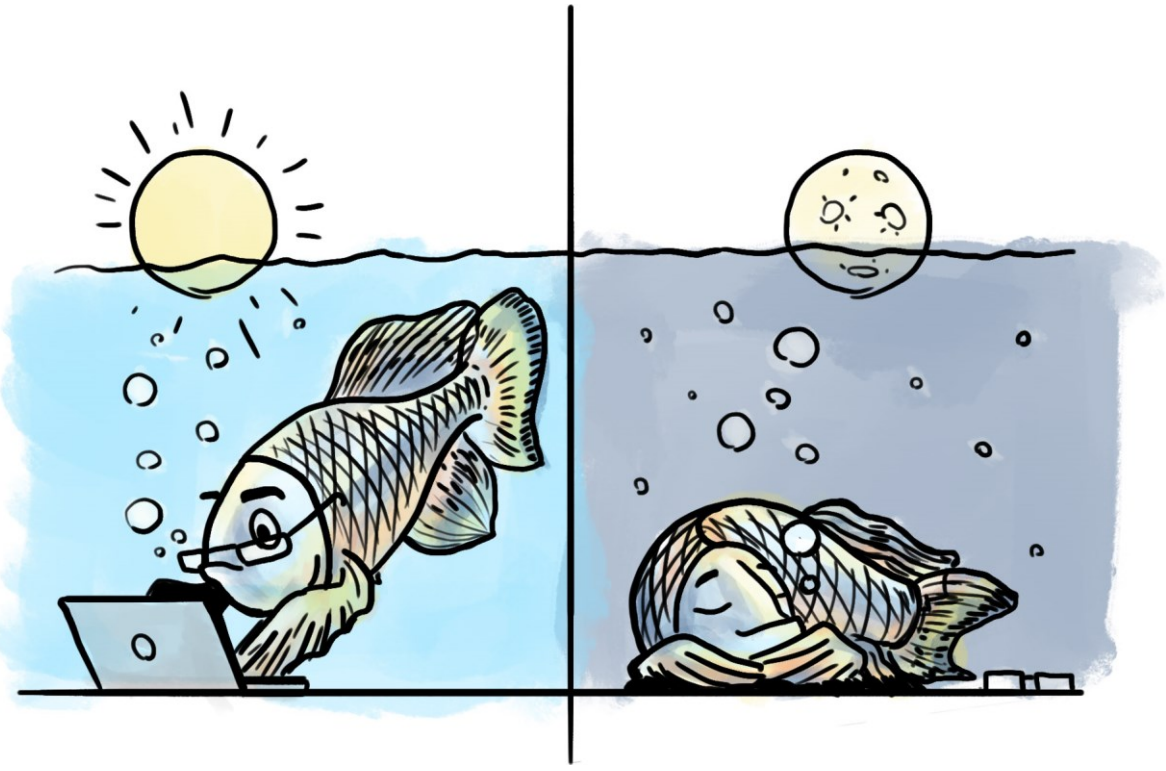
up questions related to *Nothobranchius* husbandry and senescence under laboratory conditions.

Research in natural habitats has inevitably also some pitfalls. It should be made clear that any observed absence of evidence can be biased by the effect of so-called "selective disappearance" (due to more likely death of low quality individuals from the population, Hayward et al., 2013; Hämäläinen et al., 2014). Laboratory studies that follow the lifetime reproduction of each individual separately are ideal for controlling for potential bias from selective disappearance. We therefore followed up with a laboratory project on age-dependent reproduction of individually kept males and females of *Nothobranchius furzeri*. This study (**Paper 6**) clearly demonstrated that a decline in reproductive function comes at very advanced age when only a few survivors remained in the wild populations or the pool was already desiccated. Interestingly, females suffered a larger decline in reproductive functions



than males.

Several laboratory studies in the past two decades investigated how ambient temperature affects various molecular and behavioural senescence markers of *Nothobranchius* spp. (Valenzano et al., 2006b; Hsu & Chiu, 2009; Milinkovitch et al., 2017). Nonetheless, these studies were performed at a time when no available information on the thermal requirements of this species existed. The optimal temperature was based on the experience of hobbyists (Genade et al., 2005). These studies were set at constant temperatures ignoring the conditions of periodically desiccating savanna pools with their daily temperature amplitude of 15 °C (Reichard, 2016b). This dissertation provides insights into the circadian thermal cycle and behaviour of *Nothobranchius* fish in its wild habitat (**Paper 7**). The circadian rhythm of environmental fluctuations synchronizes behaviour in populations of *Nothobranchius* spp. In the next paper (**Paper 8**) the preferred body temperatures of *Nothobranchius* spp. were assessed using a horizontal thermal gradient in the laboratory. These novel insights about environmental periodicity and thermal requirements were used for designing a crucial experiment that aimed to compare senescence patterns under the commonly used constant



temperature in laboratories and an ecologically more relevant fluctuating thermal environment (**Paper 9**). The experiment demonstrated significant improvement in survival and reproduction under the fluctuating thermal conditions.

A particular example of an incomplete framework was the utilization changes in the circadian activity of killifish with age as a senescence marker (Lucas-Sánchez et al., 2011, 2013, 2015; Almaida-Pagán et al., 2018), without knowing the natural activity pattern of wild fish. **Paper 7** demonstrated that wild *Nothobranchius* fishes were mostly active during daylight which is, in accordance with laboratory observations.

Development of standardized laboratory conditions represents both an absolute necessity and a challenge. The absence of well-defined and uniform conditions may be a contributing factor behind the recently identified replication crisis in life sciences (Ioannidis, 2005; Barnard et al., 2009). As an example, standardization of diet for animals in captivity is still a relatively neglected topic in the husbandry of many animal models (Penglase et al., 2012; Watts & D´Abramo, 2021). This is also the case for *Nothobranchius* spp. despite pioneering diet experiments that aim to modify senescence (Valenzano et al., 2006a; Terzibasi et al., 2009; Wei et al., 2020) or reproduction (Vrtílek & Reichard, 2015). *Nothobranchius* spp. in laboratory colonies are currently mainly fed with live or frozen chironomid larvae (Polačik et al., 2016; Dodzian et al., 2018; Philippe et al., 2018), a nutritionally variable source also potentially containing contaminants and pathogens (Shari et al., 2014; Reichard et al., 2022). Growth and maturity of captive *N. furzeri* is much slower than their wild counterparts (compare **Paper 3** and **Paper 9** for maturation, and **Paper 2** and **Paper 6** for growth). Both of these delayed life history traits in captivity may be related to suboptimal nutrition, and may have significant consequences for senescence (Tarry-Adkins & Ozanne, 2017). **Paper 10** provides a protocol on how to replace chironomid larvae in the diet of *N.*

furzeri with pelleted food. This is the first step in developing a standardized feeding protocol. A major benefit of pelleted food is better control of its nutritional content and opens up the opportunity for nutritional modifications.



Many studies on the histology of laboratory fish are burdened by misdiagnoses (Wolf et al., 2015, 2018). It would be surprising if the same had not happened in research using *N. furzeri*. *Nothobranchius furzeri* was thought to be extremely prone to developing tumours which is rather exceptional among fishes (Ackerman & Gerhard, 2018). Nonetheless, the pattern was conspicuous as tumour incidence was unnaturally high (Hochberg & Noble, 2017). It was therefore presumed to be a very promising model for the study of carcinogenesis (Di Cicco et al., 2011; Blažek et al., 2017). **Paper 11** offers an overview of *Nothobranchius* histopathology and identifies potential misdiagnoses appearing in the literature using *N. furzeri* as a model. This last paper of the dissertation questions the origin of neoplasias as spontaneous and interprets them as the result of an immune response towards bacterial

infections. The immune response expressed as mononuclear cell aggregates may have been misidentified as tumours in at least some papers published during the last two decades. **Paper 11** also discussed the role of cellular vacuolation in liver tissue both in captive and wild fish and to what extent its rate is detrimental to annual killifish (as suggested by Godoy et al., 2019, for example).

More detailed information regarding the above-mentioned topics can be found in the corresponding papers attached below.

PAPER 1: Overview of short-lived fishes

Žák, J., Vrtílek, M., Polačik, M., Blažek, R., & Reichard, M. (2021). Short-lived fishes: Annual and multivoltine strategies. Fish and Fisheries, 22(3), 546-561.

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ORIGINAL ARTICLE

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Short-lived fishes: Annual and multivoltine strategies

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Abstract

The diversity of life histories across the animal kingdom is enormous, with direct consequences for the evolution of lifespans. Very short lifespans (maximum shorter than 1 year in their natural environment) have evolved in several vertebrate lineages. We review short-lived fish species which complete either single (annual/univoltine) or multiple (multivoltine) generations within a year. We summarize the commonalities and particulars of their biology. Apart from annual killifishes (with >350 species), we detected 60 species with validated lifespan shorter than 1 year in their natural environment. Considering the low number of reports on fish lifespan (<5% of 30,000+ fish species; 1,543 species), the total number of short-lived fish species may be relatively high (>1,200 species). Short-lived fish species are scattered across 12 orders, indicating that short lifespan is not a phylogenetically conserved trait but rather evolves under specific ecological conditions. In general, short-lived fish species are small (typically 55 ± 35 mm), experience high predation (making them important part of ecosystem trophodynamics) and live in shallow warm waters with high productivity and stable abiotic conditions (e.g. Gobiidae, Clupeidae). Others utilize temporally constrained environments, where they survive unfavourable conditions as dormant stages (annual killifishes). They also utilize less productive environments; in this case, they migrate between productive and un-productive environments (e.g. Myctophidae, Salangidae). These species include the putatively shortest-lived (*Schindleria pietschmanni*: Schindleriidae) and earliest maturing (*Nothobranchius furzeri*: Nothobranchiidae) vertebrates and represent the lower limit of vertebrate longevity. Their examination may provide important insights into the evolutionary and mechanistic understanding of ageing.

KEYWORDS

ephemeral lifespan, extreme longevity, generation time, life history, otolith

1 | INTRODUCTION

Lifespan has a formative role on most other life-history traits as it defines the timeframe for completion of all life-determining processes (Stearns, 1992). Longevity in wild populations is the most relevant lifespan estimate from an evolutionary perspective, because it integrates all longevity-shaping forces of natural selection (Monaghan et al., 2008; Nussey et al., 2008). In particular, lifespan in natural populations is moulded by the mode and rate of extrinsic mortality (Maklakov et al., 2015; Medawar, 1952; Williams, 1957), dominated by predation, diseases, competition, natural disasters and accidental deaths (Reichard, 2017). Species with high extrinsic mortality are expected to evolve short lifespans (Medawar, 1952; Williams, 1957) and those species may provide critical insights into how natural selection limits lifespan.

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Among vertebrates, several species are reported to have extremely short lifespans of several months in their natural environment (Depczynski & Bellwood, 2005; Karsten et al., 2008). One of the first references to a short-lived vertebrate dates from the 19th century when R. Collet used the term "annual vertebrate" for a transparent goby—*Aphia minuta* (Gobiidae) (Collet, 1878). Since then, vertebrates with a maximum lifespan equal to or shorter than a year have been reported from wild populations of a whole range of vertebrate taxa including fish (Depczynski & Bellwood, 2005), reptiles (Eckhardt et al., 2017) and mammals (Dickman & Braithwaite, 1992). All these species experience strong adult mortality induced by high predation rate (Depczynski & Bellwood, 2006), environmental constraints (Berois et al., 2016; Eckhardt et al., 2017) or exhibit high postreproductive mortality (Dickman & Braithwaite, 1992). Understanding the causes and consequences of extremely short lifespans is important for conservation management (Lessa et al., 2008), didactic purposes (Flindt, 2006) and for the development of evolutionary theories of life histories (Stearns, 1992).

Production of more than a single generation per year or development of dormant stages (or both strategies) is necessary for the persistence of short-lived species. Species with multiple generations within a year are termed multivoltine species (Crawford, 1981; Southgate, 1981) and are common among invertebrate taxa (Corbet et al., 2006; Crawford, 1981; Southgate, 1981), but relatively rare in vertebrates (Lambin & Yoccoz, 2001). Using dragonflies (Odonata), Corbet et al. (2006) demonstrated that multivoltinism is positively related to low latitude, in accordance with the prediction that multivoltine species are associated with high ecosystem productivity. To our knowledge, the term "multivoltine" is applied rarely (if ever) to ray-finned fishes (Actinopterygii). We believe that the term "multivoltine species" is appropriate for fish species fulfilling the criterion of multiple generations per year.

Ray-finned fishes are the most diversified vertebrate group with the occurrence of short lifespans. Among them, killifishes (family Nothobranchiidae in Africa and Rivulidae in the Neotropics, Cyprinodontiformes) are apparently the most diverse group of short-lived fishes, containing more than 270 and 420 described species respectively (Froese & Pauly, 2019) of which 350 species (i.e. approximately 50%) are so-called annual species (Huber, 2020). In annual killifishes, posthatching lifespan is supposedly shorter than a year, because they occur almost exclusively in regions where seasonal rainfall gives rise to temporary pools which desiccate within several months (Berois et al., 2016; Furness, 2016; Reichard & Polačik, 2019). Indeed, the duration of pool inundation is the main evolutionary force determining the evolution of annual killifish lifespan—species and populations from drier regions retain shorter lifespans in captivity (Blažek et al., 2017; Terzibasi et al., 2008). When their pool desiccates, all adults die and populations persist only as desiccation-resistant embryos buried in dry mud (Furness, 2016). The short lifespan and suitability for captive breeding have made annual killifishes a promising model for ageing research (Cellerino et al., 2016; Walford, 1969). Their high diversity and general awareness of their short lifespan contribute to the almost exclusive use of the term "annual fish" for annual killifish (Berois et al., 2016; Myers, 1952; Wourms, 1967), despite the fact that annual lifespan is not exclusive to annual killifish.

Paedomorphic species are another abundant group among exceptionally short-lived fishes (Kon & Yoshino, 2002; La Mesa, 1999, this study). Progenetic paedomorphosis represents a condition when maturation of the gonads is completed while somatic characters remain in a nonadult state (La Messa, 2011). In ray-finned fishes, this phenomenon is associated with body miniaturization (Johnson & Brothers, 1993; Rüber et al., 2007). For example, *Schindleria* spp. are very small (< 22 mm) scaleless gobies, with reduced pigmentation and ossification (Johnson & Brothers, 1993). Paedomorphic fish species with known lifespan are exceptionally short-lived. This is in striking contrast to the extended lifespan of paedomorphic amphibians (Voituron et al., 2011), providing interesting potential to study the environmental conditions and evolutionary mechanisms of this difference.

To our knowledge, the occurrence of short-lived ray-finned fish species with a natural lifespan shorter than one year has not been systematically summarized. In this review, we aim (a) to provide an overview of short-lived ray-finned fish species with maximum lifespans equal to or shorter than one year in the wild. In general, we exclude reports of short lifespans of annual killifish from this review (since they have been reviewed elsewhere (e.g. Berois et al., 2016)), with exceptions where it was necessary to include them for appropriate interpretation of findings. (b) We use our unpublished results from wild populations of African annual killifish, *Nothobranchius furzeri*, to compare natural lifespans between this species and lifespan reports of other short-lived fish species. (c) We characterize the biology of short-lived fish species to determine common trends in their ecology and life history. (d) Finally, we define and characterize the terms "annual fish" and "multivoltine fish."

2 | MATERIAL AND METHODS

2.1 | Databases searched

We were principally interested in fish species that exhibit a maximum lifespan shorter than one year in their natural environment. First, we reviewed databases developed to record animal lifespans—AnAge database (Tacutu et al., 2013) accessed 30 May 2020 (https://genomics.senescence.info/species/), Longevity Records (McDonough et al., 2000) accessed 25 May 2020 (https://www.demogr.mpg.de/longevityrecords/0503.htm) and *rfishbase* package v. 3.0.4 (Boettiger et al., 2012) implemented in R environment (R Core Team, 2019) accessed 27 May 2020. Second, we evaluated the comprehensiveness of available fish species lifespan information using *rfishbase* to estimate the proportion of ray-finned fish species with reported maximum lifespan overall. Given that references in the databases often cited sources prone to imprecise data, such as "grey literature" or books, we used Google Scholar (https://scholar.google.com/) to search for the primary source of database information.

TABLE 1 Overview of shortcomings of age estimation from otolith readings based on criteria outlined by Campana (2001), and extended to include other potential biases relevant for our study

Index	Caveat
X	A different method than otolith reading was used for lifespan determination
a	Formation of daily growth increments in otolith was not validated for the studied species
b	The age at first otolith increment formation was not validated
c	The increments in outer edge of otolith show reduced readability—potential underestimate of maximum age
d	Sampling did not cover the potential for seasonal fluctuation in population age structure (i.e. sampling was performed less than three times within a year)
e	Species age estimate was based on sampling of a single population
f	Sample size was not provided, or was smaller than 100 when the whole body size spectrum per species was age determined or when less than 10 largest individuals were age determined

Note: Reported indices serve to determine possible shortcomings in data presented in Tables 2–4 and suggest where lifespan estimates may change in future when more studies have been conducted. The reported data on species lifespan in Tables 2–4 should be taken as an evidence of lifespan shorter than one year rather than as a precise estimate of maximum natural lifespan.

We used species Latin name in quotation marks and then added one of the following five search terms: *longevity*, *lifespan*, *life span*, *age*, *otolith*. Each term was used for a separate search. For each run, we checked the first 260 short text snippets/abstracts provided by Google Scholar. For relevant entries, we screened the source article to validate original information. We also searched those articles for any link to other records of short-lived fish species.

Third, we searched keyword "annual fishes" in Google Scholar (excluding patents and citations, accessed 8 June 2020) and checked the first 800 results out of a total of 1,360. Of the first 800 results 24 were irrelevant and were omitted. From the rest, 97.7% referenced annual killifish only. The term "annual fishes" was used instead of "annual fish" because the latter term provided only 2% of results related to short-lived fish species other than annual killifish (7 out of 300 checked results, 11,800 results in total). 57% of results (172 out of 300) related to annual killifish and 40% (121 of 300) yielded irrelevant results such as "statistics of annual fish harvest" etc. A search for the term "ephemeral fish" (65 results) returned almost exclusively nonrelevant results and, in a few cases, it returned the species already yielded by the previous searches.

2.2 | Criteria for suitable longevity report

The retrieved records were screened using the following criteria. The major criterion was that it had been externally peer-reviewed or published as a report of a relevant international authority (e.g. Food and Agriculture Organization of the United Nations, Australian Centre for International Agricultural Research or South Pacific Commission). In addition to peer-reviewed articles and reports, three theses/dissertations were also included as relevant resources.

The second criterion for selection was the method for age estimate. The following means of maximum lifespan estimates were considered reliable, reading of daily growth increments in otoliths (Pannella, 1971), following survival of individuals in the wild (Stearns, 1983) and duration of habitat existence in species from ephemeral habitats (Pen et al., 1993; Vrtílek et al., 2018). Given the variation in the quality of reported results, we ascribe notes to each reported maximum age estimate (Table 1) to pinpoint potential methodological issues (Campana, 2001).

Several studies used age estimating methods that did not fulfil our criteria and were thus omitted. Studies that estimated age using body size distributions were omitted, because age may be decoupled from body size when fish reach asymptotic body size (Choat & Robertson, 2002; O'Farrell et al., 2015). Reports that simply mentioned species maximum lifespan, but did not provide relevant information how the estimate was reached, were excluded from the main body of the paper and are summarized in Table S1. Some species have principally annual populations while other natural populations live longer than one year, such as *Gasterosteus aculeatus* (Gasterosteidae) (Giles, 1987) or *Rhodeus amarus* (Cyprinidae) (Konečná & Reichard, 2011). These species are not part of this review because they often live longer than one year in the wild. Similarly, species with sexual dimorphism in lifespan and only one of the sexes having a maximum lifespan of less than one year in the wild, such as *Gambusia* spp. (Poeciliidae) (Cabral & Marques, 1999) or *Ceratoscopelus warmingii* (Myctophidae) (Linkowski et al., 1993), were not included.

3 | RESULTS AND DISCUSSION

3.1 | Overview of short-lived fish species and their phylogenetic position

We found 60 species (Tables 2–4) other than annual killifish with reported maximum lifespan of one year or less in their natural environment. These species are distributed over 12 orders (Figure 1) suggesting that short lifespan has evolved independently several

TABLE 2 Overview of short-lived fish species from order Gobiiformes with natural lifespan shorter than one year

Species	Order (Family)	Voltinism	Largest specimen	Max. lifespan	Major source of mortality	Reference
Aphia minuta	Gobiiformes (Gobiidae)	Annual	55 mm TL	275 days[b]	Reproduction	Iglesias et al. (1997), La Mesa (1999)
Crystalogobius linearis	Gobiiformes (Gobiidae)	Annual	41 mm TL	225 days[a,b,e]	Reproduction	La Mesa (2001)
Eviota sigillata	Gobiiformes (Gobiidae)	Multivoltine	18 mm TL	59 days[b,d]	Predation	Depczynski and Bellwood (2005)
Eviota melasma	Gobiiformes (Gobiidae)	Multivoltine	27 mm TL	97 days[b,d]	Predation	Depczynski and Bellwood (2006)
Eviota queenslandica	Gobiiformes (Gobiidae)	Multivoltine	26 mm TL	99 days[b,d]	Predation	Depczynski and Bellwood (2006)
Eviota epiphanes	Gobiiformes (Gobiidae)	Multivoltine	19 mm TL	61 days[b,d,e,f]	Predation	Grant (2013); Longenecker and Langston (2005)
Trimma nasa	Gobiiformes (Gobiidae)	Multivoltine	17 mm SL	87 days[a,b,d,e]	Predation	Winterbottom and Southcott (2008)
Trimma benjamini	Gobiiformes (Gobiidae)	Multivoltine	23 mm SL	140 days[a,b,d,e,f]	Predation	Winterbottom et al. (2011)
Paedogobius kimurai	Gobiiformes (Gobiidae)	Multivoltine	16 mm SL	67 days[a,b,e,f]	Reproduction, predation	Kon and Yoshino (2002)
Mugilogobius chulae	Gobiiformes (Gobiidae)	Multivoltine	21 mm SL	137 days[c,e,f]	Predation	Kunishima and Tachihara (2019)
Mugilogobius sp.	Gobiiformes (Gobiidae)	Multivoltine	36 mm SL	150 days[c,e,f]	Predation	Kunishima and Tachihara (2019)
Istigobius decoratus	Gobiiformes (Gobiidae)	Annual	84 mm TL	266 days[c]	Predation	Kritzer, (2002)
Valenciennea muralis	Gobiiformes (Gobiidae)	Annual	111 mm TL	363 days[b,e]	Predation	Hernaman and Munday (2005)
Pseudogobius masago	Gobiiformes (Gobiidae)	Multivoltine	18 mm SL	124 days[b]	NA	Saimaru et al. (2018)
Brachygobius mekongensis	Gobiiformes (Gobiidae)	Multivoltine	14 mm SL	78 days[a,b]	NA	Morioka and Sano (2009)
Coryphoterus kuna	Gobiiformes (Gobiidae)	Multivoltine	15 mm SL	148 days[a,b,f]	Predation	Victor et al. (2010)
Coryphopterus personatus/hyalinus	Gobiiformes (Gobiidae)	NA	58 mm TL	195 days[a,b,d,e,f]	Predation	Beeken (2019)
Bathygobius coalitus	Gobiiformes (Gobiidae)	Annual	68 mm TL	307 days[b,c,e]	NA	Shafer (2000)
Schindleria sp.	Gobiiformes (Schindleridae)	Multivoltine	30 mm SL	60 days[a,b,e]	Reproduction, predation	Kon and Yoshino (2002)
Schindleria praematura	Gobiiformes (Schindleridae)	Multivoltine	24 mm TL	42 days[a,b,f]	Reproduction, predation	Landaeta (2002(; Whittle (2003)
Schindleria pietschmanni	Gobiiformes (Schindleridae)	Multivoltine	17 mm TL	29 days[a,b]	Reproduction, predation	Whittle (2003)

Note: Taxonomic group is assigned in accordance with Rabosky et al., 2018. Voltinism is considered as: "annual"—species with lifespan one year or shorter, which probably complete a single generation within a year , "multivoltine"—species complete two or more generations within a year (Corbet et al., 2006). The largest specimen refers to the size of the largest age determined individual, TL = total length, SL = standard length. Superscript letters for lifespan records are defined in Table 1. Age reports with more indices have higher likelihood of longevity estimate changing in the future. Common names of all listed species are given in Table S2.

times. Data on maximum fish age are generally scarce, because only 4.7% (1,543 of a total of 32,872 species) of extant ray-finned fish species have registered lifespans in *rfishbase*. In general, short-lived species are small with body size 55 ± 35 mm (median ± median absolute deviation, both rounded to 5 mm). Below, we provide definitions of annual and multivoltine fish and synthesize the evolutionary ecology and basic biological characteristics of the most commonly reported species.

3.2 | Biology of the most frequently reported groups of short-lived fish

3.2.1 | Short-lived gobies

The second largest group of fish species (after annual killifishes) with reported lifespan shorter than a year in our literature review were short-lived gobies (Gobiiformes) (Table 2). Short-lived gobies

TABLE 3 Overview of short-lived fish species from orders Clupeiformes and Myctophiformes with natural lifespan shorter than one year

Species	Order (Family)	Voltinism	Largest specimen	Max. lifespan	Major source of mortality	Reference
Spratelloides robustus	Clupeiformes (Clupeidae)	Annual	82 mm FL	241 days[b]	Predation	Rogers et al. (2003)
Spratelloides delicatulus	Clupeiformes (Clupeidae)	Multivoltine	66 mm SL	159 days[b]	Predation	Dalzell et al. (1987), Milton and Blaber (1993), Milton et al. (1991), Milton et al. (1993), Milton et al. (1989)
Spratelloides lewisi	Clupeiformes (Clupeidae)	Multivoltine	63 mm SL	179 days[a,b]	Predation	Milton et al. (1991)
Spratelloides gracilis	Clupeiformes (Clupeidae)	Multivoltine	58 mm SL	149 days[b]	Predation	Milton and Blaber (1993), Milton et al. (1991), Milton et al. (1989)
Clupeichthys aesarnensis	Clupeiformes (Clupeidae)	Annual	60 mm SL	243 days[a,b]	Predation	Morioka et al. (2019)
Stolephorus carpentariae	Clupeiformes (Eugraulidae)	Multivoltine	71 mm TL	158 days[b,e,f]	Predation	Hoedt (2002)
Stolephorus nelsoni	Clupeiformes (Eugraulidae)	Annual	103 mm TL	285 days[b]	Predation	Hoedt (1989, 2002)
Stolephorus commersonnii	Clupeiformes (Eugraulidae)	Annual	158 mm TL	240 days[b,c,e,f]	Predation	Hoedt (2002)
Stolephorus insularis	Clupeiformes (Eugraulidae)	Multivoltine	64 mm TL	126 days[b,e,f]	Predation	Hoedt (2002)
Encrasicholina heteroloba	Clupeiformes (Eugraulidae)	Annual	90 mm TL	240 days[b,c]	Predation	Milton and Blaber (1993), Milton et al. (1989), Wright et al. (1989)
Encrasicholina devisi	Clupeiformes (Eugraulidae)	Multivoltine	62 mm SL	122 days[b]	Predation	Hoedt (2002), Milton and Blaber (1993)
Encrasicholina purpurea	Clupeiformes (Eugraulidae)	Multivoltine	63 mm SL	189 days[b]	Predation	Struhsaker and Uchiyama (1976)
Encrasicholina punctifer	Clupeiformes (Eugraulidae)	Multivoltine	80 mm SL	114 days[b,f]	Predation	Milton and Blaber (1993)
Engraulis japonicus	Clupeiformes (Eugraulidae)	Annual	137 mm SL	329 days[b]	Predation	Yukami et al. (2008)
Benthosema suborbitale	Myctophiformes (Myctophidae)	Annual	33 mm SL	325 days[b]	Predation, reproduction	Gartner (1991)
Benthosema pterotum	Myctophiformes (Myctophidae)	Annual	53 mm SL	300 days[a,b]	Predation, reproduction	Gjøsæter (1981, 1984)
Benthosema fibulatum	Myctophiformes (Myctophidae)	Annual	83 mm SL	300 days[a,b,d,f]	Predation, reproduction	Gjøsæter (1978, 1981)
Diaphus dumerilii	Myctophiformes (Myctophidae)	Annual	63 mm SL	360 days[b,]	Predation, reproduction	Gartner (1991)
Symbolophorus evermanni	Myctophiformes (Myctophidae)	Annual	86 mm SL	249 days[a,b,d,e,f]	Predation, reproduction	Gjøsæter (1987)
Myctophum spinosum	Myctophiformes (Myctophidae)	Annual	81 mm SL	302 days[a,b,d,e,f]	Predation, reproduction	Gjøsæter (1987)

Note: Taxonomic group is assigned in accordance with Rabosky et al., 2018. Voltinism is considered as "annual"—species with lifespan one year or shorter, which probably complete a single generation within a year, "multivoltine"—species complete two or more generations within a year (Corbet et al., 2006). The largest specimen refers to the size of the largest age determined individual, TL = total length, SL = standard length, FL = fork length. Superscript letters for lifespan records are defined in Table 1. Age reports with more indices have higher likelihood of longevity estimate changing in the future. Common names of all listed species are given in Table S3.

TABLE 4 Overview of a short-lived fish species from less frequently reported orders

Species	Order (Family)	Voltinism	The largest specimen	Max. lifespan	Major source of mortality	Reference
Ostorhinchus rubrimacula	Kurtiformes (Apogonidae)	Annual	44 mm SL	274 days[a,b,d,e]	Predation	Longenecker & Langston, 2006)
Rhabdamia gracilis	Kurtiformes (Apogonidae)	Annual	46 mm SL	322 days[a,b,f]	Predation	Dalzell et al. (1987), Milton and Blaber (1993)
Rhabdamia cypselurus	Kurtiformes (Apogonidae)	NA	45 mm SL	105 days[a,b,f]	Predation	Milton and Blaber (1993)
Apogon erythrinus	Kurtiformes (Apogonidae)	NA	NA	120 days[a,b,d,e,f]	Predation	Longenecker and Langston (2005)
Plecoglossus altivelis	Osmeriformes (Plecoglossidae)	Annual	172 mm SL	355 days[c]	Reproduction	Tsukamoto and Kaiihara, (1987)
Salanx ariakensis	Osmeriformes (Salangidae)	Annual	157 mm SL	354 days[b,d,e,f]	Reproduction	Yuhao et al. (2001)
Neosalanx taihuensis	Osmeriformes (Salangidae)	Annual	75 mm SL	<365[a,b,c,e]	Reproduction	Wu et al. (2011)
Rasbora rubrodorsalis	Cypriniformes (Cyprinidae)	Multivoltine	31 mm SL	121 days[a,b]	NA	Morioka et al. (2014)
Esomus metallicus	Cypriniformes (Cyprinidae)	Multivoltine	47 mm SL	118 days[a,b]	NA	Morioka and Vongvitchith (2014)
Tanichthys albonubes	Cypriniformes (Cyprinidae)	Multivoltine	NA	130 days[e,f]	NA	Shi et al. (2008)
Engraulicypris sardella	Cypriniformes (Cyprinidae)	Annual	112 mm TL	251 days[c]	Predation, reproduction	Rufli and van Lissa (1982; Thompson and Bulirani (1993)
Galaxiella nigrirostrata	Galaxiiformes (Galaxiidae)	Annual	48 mm TL	<365 days[X]	Reproduction, habitat duration	Pen et al. (1993)
Galaxiella pusilla	Galaxiiformes (Galaxiidae)	Annual	35 mm TL	<365 days[X]	Reproduction, habitat duration	Humphries (1986)
Enneapterygius atriceps	Blenniformes (Trypterigiidae)	Multivoltine	26 mm SL	117 days[a,b,f]	Predation	Longenecker and Langston (2005)
Salarias patzneri	Blenniformes (Blenniidae)	Annual	59 mm TL	340 days[b,e]	Predation	Wilson (2004)
Parambassis siamensis	Ovalentaria (Ambassidae)	Multivoltine	41 mm SL	148 days[b]	NA	Okutsu et al. (2011)
Vinciguerria nimbaria	Stomiiformes (Phosichtidae)	Annual	56 mm SL	240 days[a,b]	Predation, reproduction	Stequert et al. (2003); Tomás and Panfili (2000)
Heterandria formosa	Cyprinodontiformes (Poeciliidae)	Multivoltine	NA	120 days[a,b,e,f]	Predation	Soucy and Travis (2003)
Hypoptychus dybowskii	Perciformes (Hypoptychidae)	Annual	78 mm SL	365 days[e]	Reproduction, predation	Narimatsu and Munehara (1997)

Note: Taxonomic group is assigned in accordance with Rabosky et al., 2018. Voltinism is considered as "annual"—species with lifespan one year or shorter, which complete a single generation within a year, "multivoltine"—species complete two or more generations within a year (Corbet et al., 2006). The largest specimen refers to the size of the largest age determined individual, TL = total length, SL = standard length. Superscript letters for lifespan records are defined in Table 1. Age reports with more indices have higher likelihood of longevity estimate changing in the future. Common names of all listed species are given in Table S4.

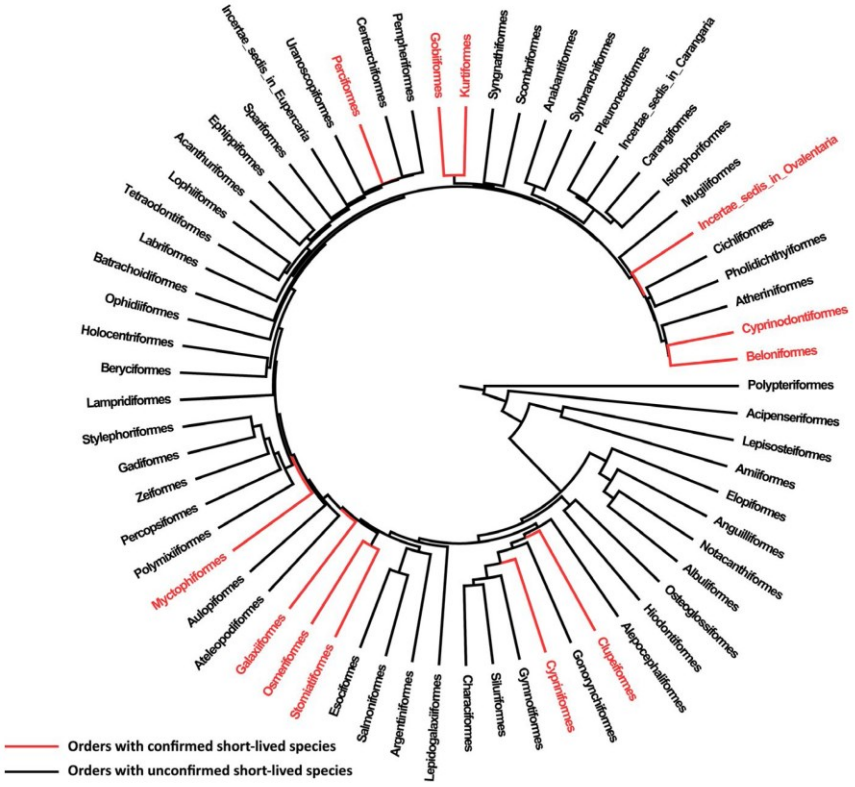


FIGURE 1 Distribution of short-lived (annual or multivoltine) fish species within Actinopterigian fish orders. Orders with confirmed presence of short-lived fish species are in red. Orders without confirmed presence of short-lived fish species are in black. Primary source of data is Rabosky et al., 2018https://fishtreeoflife.org/taxonomy/. Figure was produced by Fig tree software v 1.4.4. (Rambaut, 2018)

include both marine and freshwater species (Morioka & Sano, 2009; Winterbottom et al., 2011), and bottom dwelling and pelagic species (Depczynski & Bellwood, 2006; Iglesias et al., 1997). Paedomorphic species are common in this group, for example *Paedogobius kimurai* (Gobiidae), (Kon & Yoshino, 2002) or *Schindleria* sp. Those species are estimated to produce nine generations per year (Kon & Yoshino, 2002). This is the highest number of generations produced per year among fish species, and conceivably among all vertebrates. The vast majority of the known short-lived goby species inhabit tropical coral reefs, where they represent an important part of trophic network, feeding on detritus and zooplankton and being predated by piscivores (Brandl et al., 2019; Wilson, 2004; Winterbottom & Southcott, 2008). Given their small body size, the predation pressure can become a major constraint for their lifespan (Depczynski & Bellwood, 2006; Winterbottom & Southcott, 2008). Most of the short-lived goby species reproduce repeatedly and retain the male parental care typical for other gobies (Depczynski & Bellwood, 2006; Patzner et al., 2011).

3.2.2 | Short-lived clupeids and myctophids

Clupeids (Clupeiformes) with a maximum lifespan shorter than a year in the wild (Table 3) are mostly found in tropical and subtropical marine environments (Hoedt, 2002; Struhsaker & Uchiyama, 1976), but are also reported from freshwaters (Morioka et al., 2019). Short-lived clupeids are small pelagic species feeding mostly on zooplankton. The major source of adult mortality in short-lived clupeids is predation by larger pelagic fish (Blaber et al., 1990; Milton et al., 1991) and commercial fishery also significantly contributes to their mortality (Milton et al., 1991; Morioka et al., 2019). Lifespan data from the short-lived clupeids are available because of their economic importance for human consumption and as a tuna-baitfish (Milton et al., 1993). Similarly to short-lived gobies, short-lived clupeids rarely reach their asymptotic body size and their growth remains linear throughout their natural lifespan in the wild (Rogers et al., 2003; Struhsaker & Uchiyama, 1976). This may be a result of intensive predation, restricting them from reaching asymptotic body size. Alternatively, explosive growth may be a strategy to increase fecundity (Barneche et al., 2018; Williams, 1966; Winemiller & Rose, 1993) and escape increased mortality at small size (Meekan et al., 2006; Miller et al., 1988). Mature short-lived clupeids spawn repeatedly in open water at intervals of several days (Clarke, 1987; Rogers et al., 2003).

Lantern fishes (Myctophiformes) are small bioluminescent species inhabiting mesopelagic and benthopelagic habitats (Catul et al., 2011). They perform diurnal migration and occupy resource-rich epipelagic waters at night (Gartner, 1991). Despite spending a large amount of time in cold and resource-poor deep waters, their life-history traits are defined by epipelagic conditions, as their early development, feeding and reproduction take place in the epipelagic zone (Catul et al., 2011; Gartner, 1991). The lantern fishes with lifespans shorter than a year are from warm-water regions and their distribution only borders temperate zones (Table 3). Lantern fishes contribute to the transfer of energy from the epipelagic habitat to deeper ocean zones by their diurnal migrations (Catul et al., 2011).

They constitute an important food resource for marine predators (Cherel et al., 2010) and experience a similarly short lifespan to pelagic clupeids (Table 3).

3.2.3 | Rarely reported groups of short-lived fish species

Several ray-finned fish orders may contain many species with maximum lifespans shorter than a year in the wild, but there is a paucity of reports given their limited access, low economic value and lower attractivity for applied and fundamental research. One noteworthy group is Asian Osmeriformes (especially families Plecoglossidae and Salangidae) – small fish species with amphidromous and landlocked populations (Iguchi, 1996; Wu et al., 2011). These species are semelparous and adults die soon after spawning (Iguchi, 1996; Wu et al., 2011). Spawning is observed twice per year, because they comprise both autumn-spawning and spring-spawning populations (Iguchi, 1996; Wu et al., 2011). Most salangids appear to have maximum lifespans shorter than a year (Shouzeng & Dagang, 1994). Unfortunately, rigorous examinations are scarce or inaccessible to us, because they are published in national languages.

Some Galaxiiformes from Australia are confirmed to have maximum lifespans shorter than a year (Humphries, 1986; Pen et al., 1993). Specifically, two small species *Galaxiella nigristriata* (Galaxiidae) and *Galaxiella pusilla* (Galaxiidae) (Humphries, 1986; Pen et al., 1993) live in rivers, creeks and seasonally desiccating pools in river alluvia (Coleman et al., 2015; Humphries, 1986). These species are able to survive habitat desiccation for an extended period of time in a wet mud or under logs and rocks (Coleman et al., 2015). They reproduce repeatedly at several-day intervals and die soon after maturity (Pen et al., 1993). This suggests that spawning effort combined with habitat duration contribute significantly to their mortality (Humphries, 1986; Pen et al., 1993).

We found very few records of short lifespan from other fish orders (Table 4). For example, small species of seahorses (Syngnathiformes) are expected to have a maximum lifespan shorter than a year but, to our knowledge, no data from the wild are available. To our knowledge, there is only one frequently cited work which suggests *Hippocampus zosterae* (Syngnathidae) as having an annual lifespan (Strawn, 1958), but this study was not designed to specifically estimate longevity or maximum lifespan of this remarkable fish. Seahorse otoliths are a poor marker of age which complicates rigorous records of their natural lifespan (Do et al., 2006). It is surprising that we could not find any verified record of a short-lived characid (Characiformes). The ecology of many characids is similar to that of the short-lived fish species described above – they are small and live in resource-rich shallow waters with high predation pressure (Weitzman & Vari, 1988), which are apparently ideal conditions for the origin and maintenance of an annual (or shorter) lifespan. We believe that our failure to document quantification of their apparently short lifespan is related to the paucity of data from the core of their distribution. Alternatively, we may have missed some reports, especially those published in local languages. There is also a relatively high number of species from other taxa where maximum lifespan shorter than a year is generally assumed but rigorous evidence is missing (Table S1). We believe that there are potentially many other species within the genera mentioned in Tables 2–4 which can also have a maximum lifespan of less than a year.

3.3 | Delimitation of annual and multivoltine fish species

It is customary to refer to short-lived fish species with a lifespan shorter than one year as "annual fish" (Berois et al., 2016; Liu et al., 2000; Myers, 1952; Wourms, 1967). In accordance with Etymonline.com ("Etymonline.com," 2020) and the Cambridge Dictionary ("Cambridge Dictionary," 2020), "annual" means something "happening once a year." This is undoubtedly the case for fish species with maximum lifespans longer than half a year (Table 2–4) and most appropriate for fish species with a life cycle that follows seasonal dynamics with one-year periodicity. We prefer to call these species "annual" rather than "univoltine" (having a single generation per year, Corbet et al., 2006) but these terms could be used interchangeably depending on the context (lifespan—annual, reproduction—univoltine).

Several fish species complete multiple generations within a year and it is therefore difficult to term them annual species. This has been argued by Wilson J. E. M. Costa earlier and has led to his redefinition of *Nematolebias* (Rivulidae) Costa, 2002 from "annual killifish" to "seasonal killifish" (Costa, 2002). "Seasonal fish" is another term coined for killifish from seasonal pools (Pienaar, 1968), albeit with rare use in the literature. "Seasonality" in the term "seasonal fish/killifish" correctly reflects the exclusively temporary occurrence of these species during the rainy season.

Many short-lived fish species complete their generations irrespective of any seasonality (Tables 2–4). For example *Schindleria* sp. occurs in tropical waters and it has been estimated that they complete up to nine generations within a year (Kon & Yoshino, 2002). *Eviota* spp. (Gobiidae) or *Paedogobius kimurai* can complete up to seven generations (Depczynski & Bellwood, 2006; Kon & Yoshino, 2002) and *Encrasicholina dewisi* (Engraulidae) can complete three generations per year (Hoedt, 2002; Milton & Blaber, 1993). More species with similar life histories are listed in Tables 2–4. For these fish species, we suggest using the term "multivoltine fish." Voltinism refers to the number of generations (from birth to sexual maturity) produced by an organism per year. This term enables us to separate short-lived species into univoltine (limited to a single generation per year) and multivoltine (capable of completing more than one generation per year) (Corbet et al., 2006). Voltinism is a common concept in entomology (Corbet et al., 2006; Southgate, 1981) and has also been used for short-lived mammals (Lambin & Yoccoz, 2001). We note that generations of a multivoltine species may be distinct (nonoverlapping) or overlapping (Bjørnstad et al., 2016). We encourage the use of terms "annual" (or "univoltine" when appropriate due to focus on the generation turnover) for short-lived fish species which complete their life

cycle and produce a single generation within a year. We propose the use of term "multivoltine" in relation to short-lived fish species with multiple generations (from hatching to maturity) within one year.

3.4 | Evolutionary ecology of short-lived fish species

3.4.1 | Environment

Typical environments inhabited by short-lived fish species are tropical or subtropical shallow warm waters with high productivity and high predation rates. For example, short-lived clupeids live and reproduce in warm resource-rich pelagic waters (Milton et al., 1995) and short-lived gobies are mostly found on coral reefs (Depczynski & Bellwood, 2006). Year-round high productivity and warm water support stable food resources for early developmental stages of short-lived species occurring throughout the year (Kon & Yoshino, 2002; Morioka et al., 2014). Such conditions also support rapid developmental rates and early maturity (Munch & Salinas, 2009). Highly productive environments possess high biodiversity, often maintained by high predation pressure on early life stages (Caley, 1993; Connell, 1978). Short-lived fish species are small-bodied and are predated throughout their lives, truncating their lifespan (Caley, 1993; Depczynski & Bellwood, 2006).

Short-lived species from seasonal or resource-poor environments have developed coping mechanisms to deal with periods of unfavourable conditions. For example, annual killifish survive harsh periods as dormant embryos in dry mud (Furness, 2016). An alternative strategy for short-lived fish species is migration between productive and less productive environments (Gross et al., 1988). For example, myctophids undertake diurnal migration between deep and resource-poor mesopelagic and resource-rich epipelagic zones (Gartner, 1991). Adults of Asian amphidromous species spawn in rivers but juveniles feed in resource-rich marine and brackish waters (Gross et al., 1988; Shouzeng & Dagang, 1994). The absence of fish species with maximum lifespans of less than a year in cold climates and deep-water environments is likely a real phenomenon rather than simply due to a lack of verified records, because cold environments do not appear to support life histories with a short lifespan (Munch & Salinas, 2009).

3.4.2 | Reproduction

A short lifespan produces strong selective pressure for early maturity (Stearns, 1992; Williams, 1957). *Nothobranchius furzeri* matures at the age of 14 days posthatching in the wild (Vrtílek et al., 2018). In the paedomorphic *Schindleria pietschmanni*, adults have been confirmed at the age of 18 days (Whittle, 2003). Dwarf gobies from coral reefs are reported to mature within 1–2 months (Depczynski & Bellwood, 2006). In contrast, the maturity of semelparous species is postponed and mostly coincide with the maximum lifespan (Iguchi, 1996; Shouzeng & Dagang, 1994).

Short-lived species are expected to invest heavily in reproduction to assure its success before death (Gunderson, 1997; Williams, 1966). Investment can be realized by offspring quantity or intensive parental investment into survival of a small number of offspring. These divergent strategies are both common in short-lived fish species. Parental investment includes livebearing as in *Heterandria formosa* (Poeciliidae) (Soucy & Travis, 2003), mouthbrooding as in *Rhabdamia gracilis* (Apogonidae) (Fowler & Bean, 1930), and egg fanning and paternal nest guarding in marine gobies (Sunobe, 1998). In contrast, pelagic short-lived clupeids (Milton & Blaber, 1993), small tropical freshwater cyprinids (Morioka et al., 2014; Morioka & Vongvitchith, 2014) and Australian galaxiiforms (Humphries, 1986; Pen et al., 1993) repeatedly lay batches of eggs and do not invest in parental care. Semelparous Asian salangids invest in a single reproductive bout (Shouzeng & Dagang, 1994). The diversity of these reproductive strategies in short-lived species likely arises from the diversity of environments they inhabit (Winemiller & Rose, 1993), from seasonal savanna pools to ocean mesopelagic zones (Gartner, 1991; Reichard & Polačik, 2019), and from phylogenetic signals.

3.4.3 | Body size

Small species are generally assumed to have a short lifespan (Goatley & Bellwood, 2016; Hatton et al., 2019). Body size of the confirmed short-lived fish species in the current review spans from 14 mm in *Brachygobius mekongensis* (Gobiidae) to 220 mm in the piscivorous *Austrolebias elongatus* (Rivulidae) (Alonso et al., 2020). On the other hand, higher mortality in small species is not universal because various factors such as the use of shelters (Munday & Jones, 1998) and experience with predators (Goatley & Bellwood, 2016) varies among species. The relationship between body size and longevity is complex, but we confirm that short-lived fish species are generally small.

Small size may have ecological and metabolic benefits which contribute to maintenance of small body size in some species. Small species can use a wider range of shelters (Hernaman & Munday, 2005; Munday & Jones, 1998), have lower probability of being detected by predators (Goatley & Bellwood, 2009), may utilize niches inaccessible to large fish species (Munday & Jones, 1998), can maintain denser populations per area than larger species (Ackerman et al., 2004). They also have relatively lower metabolic demands and their digestive tract can be more easily filled (Ackerman et al., 2004). The metabolic benefits of small body size may be the mechanism behind the apparent absence of a trade-off between instantaneous growth and reproduction seen in short-lived fish species (Depczynski & Bellwood, 2006; Milton & Blaber, 1993; Winterbottom & Southcott, 2008).

3.5 | The shortest-lived fish

An extremely short lifespan is a consequence of extreme conditions such as extreme predation pressure or ephemeral environments (García et al., 2019; Reichard & Polačik, 2019; Riesch et al., 2015).

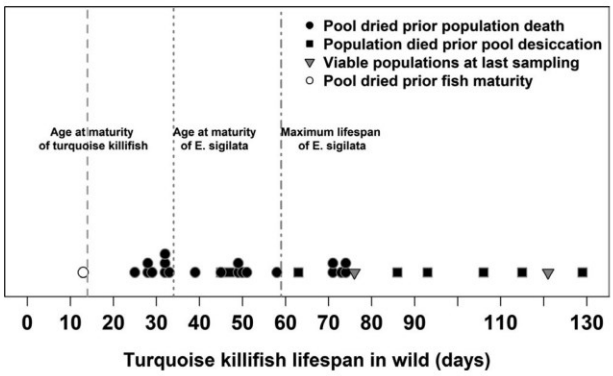


FIGURE 2 Lifespan of wild *Nothobranchius furzeri* populations in southern Mozambique. Median population lifespan is 50 days (95% confidence interval: 45–75 days). Populations inhabiting pools which dried prior to the death of all fish are depicted as black circles. Populations which disappeared prior to pool desiccation are present as black squares. Populations with inundated pools and viable populations at last sampling on 29 May 2016 (and unknown maximum lifespan) are marked as grey inverted triangles. Empty circle is a population with only juveniles. Grey vertical lines delimit common age at maturity of wild *N. furzeri*, age at maturity of *Eviota sigillata*, and maximum lifespan of *Eviota sigillata*

The relatively common occurrence of maximum lifespan shorter than one year in natural populations of ray-finned fish is unique within vertebrates.

Eviota sigillata (Gobiidae) is currently considered as the shortest-lived vertebrate with a maximum posthatching lifespan of 59 days in the wild. Our review identifies a number of other candidates for the shortest vertebrate lifespan. The most relevant is the group of small paedomorphic gobies such as *S. pietschmanni* from Hawaii (with a maximum age of 1 month assessed from 148 individuals over a 3 year sampling period (Whittle, 2003)) or a much smaller species *Schindleria brevipinguis* (<9 mm, but age not assessed (Watson & Walker, 2004)). The method of *Schindleria* spp. age estimate, however, still remains to be verified.

Among nonpaedomorphic species, the turquoise killifish *N. furzeri* from Africa is sexually mature in 2 weeks (Vrtílek, Žák, Pšenička, et al., 2018) and has a typical natural lifespan of weeks to months (Vrtílek, Žák, Polačik, et al., 2018). Prior to the report of a two-month lifespan in wild *E. sigillata* (Depczynski & Bellwood, 2005), *N. furzeri* was described as the shortest-lived vertebrate with its 3-month lifespan in captivity (Valdesalici & Cellerino, 2003). Here, we report our data from 30 wild populations of *N. furzeri* (see Supplementary methods) with a maximum lifespan from 25 to >129 days (Figure 2). Notably, 27% ($N = 8$) of wild *N. furzeri* populations with adult fish died before 34 days – the age when *E. sigillata* reaches sexual maturity (Depczynski & Bellwood, 2005). Short pool duration is not a constraint for population survival (Vrtílek, Žák, Pšenička, et al., 2018), which was supported by confirmation of viable populations in two repeatedly inundated pools which were previously inundated for less than 34 days. This is likely due to early maturity (14 days, Vrtílek, Žák, Pšenička, et al., 2018) and high daily fecundity (60 eggs per day, Vrtílek, Žák, Blažek, et al., 2018) of *N. furzeri*. These results suggest that *N. furzeri* can sustain viable populations even in more time-constrained environments and can be considered as the shortest-lived nonpaedomorphic vertebrate.

The intrinsic limits of longevity in these remarkably short-lived species can be determined by comparison with captive survival. Unfortunately, the relevant data are available only for *N. furzeri* where the maximum lifespan in captivity ranges from 3 to 17 months depending on population and holding conditions (Cellerino et al., 2016). Depczynski and Bellwood, (2006) note that *Eviota* spp. attain a longer maximum lifespan in captivity than in the wild and a captive lifespan of 2 years was reported for a closely related species *Eviota nigriventris* (Randall & Delbeek, 2009). We would like to highlight that advances in our knowledge of species with extreme life histories will very likely lead to the discovery of other fish species with an even shorter lifespan.

3.6 | Decoupling maximum lifespan from ageing

It appears that the maximum lifespan of the majority of short-lived fish species is decoupled from ageing in their natural environment. This is because their lifespan is terminated by ecological constraints, precluding sufficient time for the development of senescent changes. Thus the growth asymptote is not reached in short-lived gobies and clupeids (Depczynski & Bellwood, 2006; Struhsaker & Uchiyama, 1976) and there is no detectable reproductive senescence in wild *N. furzeri* (Vrtílek, Žák, Blažek, et al., 2018) despite their clear reproductive senescence in captivity (Žák & Reichard, 2021).

When kept in the protective environment of captivity, some species live long enough to develop senescence. It is probable that the lifespan extension induced by protective conditions in captivity is stronger in short-lived than long-lived vertebrate species (Tidière et al., 2016). Unfortunately, information related to the captive lifespan of short-lived fish species is largely anecdotal. Among gobiids, Depczynski and Bellwood, (2006) mention that *Eviota* spp. live much longer in captivity than in the wild, and that they reach the senescent stage. *Coryphopterus personatus/hyalinus* (Gobiidae) has a maximum lifespan of 195 days in the wild but can reach 4 years in captivity (Beeken, 2019). More rigorous evidence comes from short-lived cyprinodontiforms such as annual killifish (discussed in detail above) and *H. formosa*, that lives only up to 4 months in the wild (Soucy & Travis, 2003) but commonly for 14 months in captivity (Ala-Honkola et al., 2011). However, lifespan extension in captivity is far from universal. For example, benefits for semelparous species from a captive environment are negligible, because they ultimately die after reproduction (Iguchi, 1996). In addition, some short-lived fish species have a shorter maximum lifespan in captivity than in the wild or do not survive in captivity at all (e.g. *Stolephorus* spp., *Thryssa* spp. *Encrasicholina* spp., all Engraulidae (Hoedt, 2002; Milton et al., 1989)). Those short-lived species which can be kept in captivity can provide important insights into the intrinsic constrains of organismal longevity, as seen in annual killifish (Genade et al., 2005; Hu & Brunet, 2018).

3.7 | Caveats

Our knowledge of fish age is limited to a few species. Maximum lifespan is known for less than 5% of almost 33,000 described fish species (based on data from *rfishbase* (Boettiger et al., 2012; Froese & Pauly, 2019)). It is plausible that slightly more fish species have known longevity estimates, as *rfishbase* is still being developed and data from the Fish Base website (Froese & Pauly, 2019) converted to this package (Boettiger et al., 2012). This may contribute to the relatively low absolute number of reported short-lived fish species. Another caveat is that our knowledge of short lifespan is certainly biased towards economically and scientifically important species. A relatively small number of rigorous studies with small coverage over the ray-finned fish phylogeny prevents us from using a relevant phylogenetically corrected meta-analytic approach. Nonetheless, we believe that our sample size of more than 50 species makes the abovementioned generalizations on short-lived fish biology valid.

Longevity determination in short-lived species is often performed by counting the daily increments in otoliths (Pannella, 1971) and this is subject to some difficulties (see (Campana, 2001; Stevenson & Campana, 1992) for review). Thus, we list potential caveats in our overview (Table 1) which should reflect the variance in the accuracy of reported ages in Tables 2–4. In otolith readings, a higher number of increments may cause age underestimation due to problematic recognition of separate increments (Hoedt, 2002). Determination of age of first otolith increment formation is often absent in the species studied, but has only a small impact on maximum lifespan estimates, because it creates a bias of only a few days. The maximum lifespan of annual killifish from seasonal pools can be estimated from pool duration, as performed previously (Terzibasi Tozzini et al., 2013). However, annual killifish populations may disappear before the pool desiccates ((Vrtílek, Žák, Polačik, et al., 2018), Figure 2) and in such cases, the natural killifish lifespan is overestimated. In general, we have included the longevity report from studies only in species where it was generally assumed that they usually have a maximum lifespan shorter than one year in their natural environment. We note that with an increase in the number of studies related to the natural lifespan of fish species some species can be detected as longer lived than currently assumed.

3.8 | Summary

The unpreceded species richness and ecological diversity of ray-finned fish species results in a multitude of life-history strategies, including extremely short lifespans. The maximum natural lifespan of *S. pietschmanni* from Hawai is 1 month, wild *N. furzeri* populations live for 25–120+ days and *E. sigillata* lives for a maximum of 2 months. These species represent the shortest maximum lifespans among vertebrates. An important outcome of this review is that short-lived fish species are not as rare as expected. We speculate that the relatively scarce evidence of extremely short lifespan in fishes arises from a lack of interest in age determination in small fish species rather than rarity of this life history. In light of this, the unverified evidence of short-lived fish species in characiform fishes and other small tropical species is especially surprising. This contrasts with the ecological significance of short-lived fish species, which are essential in the transfer of energy in ecosystems where they occur (Brandl et al., 2019; Catul et al., 2011; Milton et al., 1991). They have economic importance as a food resource for human consumption (clupeids), are used as a baitfish (Milton et al., 1989), harvested for fishmeal production (Catul et al., 2011) and used in the ornamental fish trade (Genade et al., 2005; Randall & Delbeek, 2009). Last but not least, short-lived species which are easily bred in captivity are valuable models for evolutionary and biomedical research (Cellerino et al., 2016). We believe that our review contributes to more extensive studies into the biology and diversity of short-lived fish species.

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CONFLICT OF INTEREST

Authors declare no conflict of interests.

AUTHOR CONTRIBUTION

JZ designed the study; JZ did the literature research; JZ, MV, MR wrote the draft of manuscript; MV, JZ, MP, MR, RB collected the field data for wild lifespan of *N. furzeri*; JZ, MV, MR, MP, RB approved the final version of submitted manuscript.

DATA AVAILABILITY STATEMENT

All referenced studies included in this review can be found in the Reference section. Raw data for *Nothobranchius furzeri* lifespan are available in Figure 2. No other unique data were generated as part of this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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PAPER 2: Maturation in the wild

Vrtílek, M., **Žák, J**., Pšenička, M., & Reichard, M. (2018). Extremely rapid maturation of a wild African annual fish. Current Biology, 28(15), R822-R824.

used to conditionally alter properties of the core clock by changing cell-autonomous period, deleting TTFL function, and/ or compromising synaptic signalling. Such studies have revealed a hierarchy of neurochemically defined cell types that contribute to the correct functioning of the SCN as a network and thereby circadian behaviour. Of note are Neuromedin S-positive (NMS) cells, which constitute ca. 40% of SCN neurons. Synaptic signalling in these cells is essential for the SCN to control circadian behaviour, and changes in the properties or competence of the cell-autonomous TTFL in NMS cells are echoed at the level of mouse behavioural rhythms. In contrast, manipulations of AVP- or VIP-expressing cells (which are sub-groups of the NMS population) have a more modest effect. Paradoxically, however, loss of NMS itself has no obvious effect on the clockwork. Furthermore, recent developments of the intersectional approach have shown that, astonishingly, neurons are not the only active cellular participants in SCN timekeeping. Through conditional manipulations of the astrocyte TTFL, it is clear that the astrocytic clock can also control the ensemble SCN molecular clockwork and overt circadian behaviour of the mouse.

Prospect

The SCN is a unique brain area: two clusters of 10,000 cells exert global, hour-by-hour control over every vital function of the organism. It generates an ensemble circadian signal that has extreme robustness, persistence and precision (varying by ca. ±5 minutes over the 24 h cycle, i.e. 0.35% error). These emergent properties are established by the linkage between cell-autonomous and circuit-level mechanisms, such that outputs of the cellular clock become inputs to it (Figure 4B). Significant questions remain as to how individual SCN neurons generate day-to-day electrophysiological rhythms, what these rhythms mean for the SCN and how they are linked to the molecular clock. At the network level, the neurochemistry that 'glues' the SCN together is poorly understood: we have a fair grasp of the distribution and release of some individual neuropeptides, but no deep understanding of how peptides and other neurotransmitters come together to create a robustly functional network. Finally, we do not know the full hierarchy of signals within the SCN at the level of neurochemicals or cell-type and their topology, nor how these signals are directed outside of the SCN to determine organismal physiology. Time to get to work.

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Extremely rapid maturation of a wild African annual fish

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Ephemeral habitats can impose challenging conditions for population persistence. Survival strategies in these environments can range from high dispersal capacity to the evolution of dormant stages able to tolerate a harsh environment outside the temporal window of favourable conditions [1]. Annual killifish have evolved to live in seasonal pools on the African savannah and display a range of adaptations to cope with an unpredictable environment [2,3]. For most of the year, killifish populations survive as diapausing embryos buried in dry sediment. When savannah depressions fill with rainwater, the fish hatch, grow rapidly and, after attaining sexual maturity, reproduce daily [2,4]. *Nothobranchius furzeri*, a model species in ageing research [2,3], is distributed in a region where the climate is particularly dry and rains are unpredictable [5]. Here, we demonstrate that the fast juvenile growth and rapid sexual maturation shown by *N. furzeri* in captivity is actually an underestimate of their natural developmental rate. We estimated the age of *N. furzeri* in natural populations by counting daily-deposited increments in the otoliths and performing histological analysis of gonads. We found that *N. furzeri* are capable of reaching sexual maturity within 14 days after hatching, which to our knowledge is the fastest rate of sexual maturation recorded for a vertebrate. We also demonstrate that *N. furzeri* can grow from an initial length of 5 mm up to 54 mm over the course of a two-week period. Such rapid juvenile development is likely to be adaptive since some pools were entirely desiccated 3–5 weeks after filling, but retained a viable killifish population that reproduced before the adults succumbed to the disappearance of their pool.

We surveyed natural populations of *N. furzeri* across its range in southern

Mozambique between January and May 2016 by regular inspection of field sites (Figure 1A) and installation of dataloggers. We collected *N. furzeri* from eight separate pools (A–H, Table S1, see Supplemental Information) within a period of three weeks after the pools first filled with rainwater. Comparison between the timing of pool filling (recorded during our presence at sites and estimated from dataloggers) and fish age (estimated from otolith growth checks, n=26) indicated that fish hatched within the first three days after rains flooded a pool (Table S1). Histological analysis of male and female gonads of individuals aged 14–15 days (n=6 fish from 2 populations, and 8 control fish) demonstrated that both sexes were mature. Females possessed ripe ova and males had developed spermatozoa that were present in the sperm ducts (Figure 1C–E). Sexually dimorphic nuptial colouration (Figure 1B, Table S2) suggested that all fish were sexually mature in four populations (A, C–E) estimated to be 14 days old. In a further three populations (B, F and G) fish were estimated to be 12–15 days old and included a combination of juvenile and sub-adult fish. A final population (H) was comprised of 14-day-old fish that were still juveniles. Mean size of fish aged 14–15 days ranged from 18.8 to 42.7 mm in four populations, with an overall range of 17–43 mm in females and 17–54 mm in males. At the age of 17 days one male reached 63 mm (Table S2; n=260 fish measured overall).

Previous studies demonstrated that under optimal laboratory conditions, *N. furzeri* can reach sexual maturity in as few as 18 days (first mating attempts were observed 1–3 days earlier), followed by rapid senescence in functional traits and high intrinsic mortality [4], resulting in a median lifespan of only 4–6 months [3]. It was questioned, however, whether such a rapid life history was a natural feature of the species, or a consequence of laboratory selection for short lifespan combined with particularly favourable housing conditions with temperature, food supply and population density optimised for rapid growth [6]. Indeed, juvenile growth and sexual maturation are flexible in *N. furzeri* and respond strongly to changes in population density and resource availability.

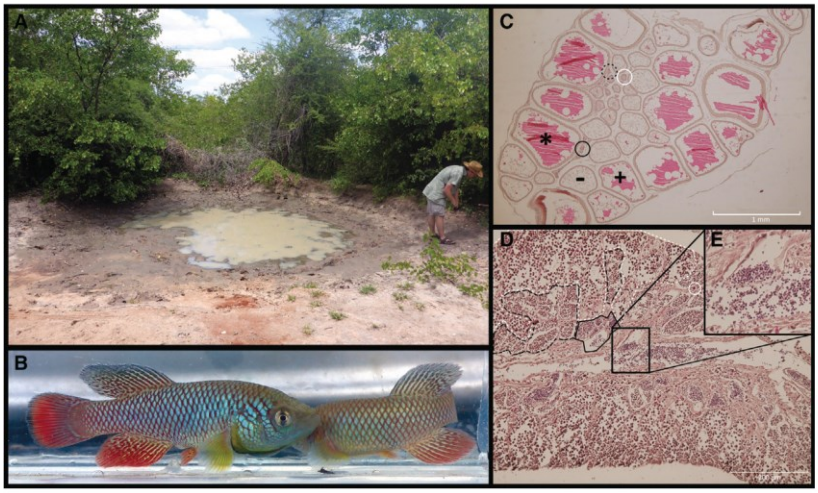


Figure 1. Adult turquoise killifish (*Nothobranchius furzeri*) and its natural habitat.
(A) Site 'D' on 27 January 2016 when it contained at least 27 adult fish estimated to be 16–17 days old. (B) Two 14-day-old male *N. furzeri* from site 'A'. (C) Histological section of an ovary from a 14-day-old female from site A that contains oogonia (dotted black circle), primary oocytes at the primary growth phase, chromatin-nucleolus stage (black circle) and perinucleolus stage (white circle), oocytes in the previtellogenic (-) and vitellogenic phase (+) and fully developed oocytes (*). (D) Histological section of the testis from a 14-day-old male from site A that includes spermatogonia (full white circle), spermatocytes (dotted white line), spermatids (broken black line) and spermatozoa (full black line). The boxed area (E) shows a magnified view of mature spermatozoa released into the spermatic duct.

Surveys of natural *N. furzeri* populations in 2016 revealed that pools typically desiccated 1–4 months after filling [7]. The shortest period for which a pool contained water and supported a killifish population that reached sexual maturity was 20 days. In that population we sampled fish at the age of 7 days (all juveniles, n=12), 14 days (adult colouration, size not measured, n=5) and 16–17 days old (all fish adult, 32–45 mm long, n=16) (Table S2). Two other pools held water (and fish) for just 33 days and the median pool duration was 65 days [7].

This finding demonstrates that fast juvenile development is adaptive in killifish despite an apparent tradeoff with early-onset ageing; even a pool that desiccated in three weeks permitted successful reproduction of *N. furzeri* and, thereby, supported a viable population that year. *Nothobranchius* species display two contrasting life-cycle phases. The first is a long-lasting embryonic period with high resistance to ageing and a bet-hedging strategy of three facultative diapause stages that spreads out the developmental time of individual embryos [2,3]; this strategy serves as an adaptation to promote survival during extended periods of drought. The second is an exceptionally short post-embryonic period characterised by rapid juvenile growth and sexual maturity — an adaptation to unpredictable pool longevity that comes with a cost of short post-embryonic lifespan [2,3].

Pronounced seasonality often gives rise to life cycles combining resting and active stages. In non-vertebrate animals, early developmental stages are usually encased in protective envelopes that shield individuals from a harsh environment. In vertebrates, it is typically the adult stage that is adapted to cope with periods of extreme conditions at seasonally dormant stages, varying from annual winter hibernation to adaptation to periodic desiccation [1]. Nevertheless, several clades of annual killifish in Africa and the Neotropics [8], along with a semelparous chameleon in Madagascar [9], have evolved annual life cycles featuring a brief post-embryonic lifespan. Among these taxa, the rapid post-hatching development and maturation shown by *N. furzeri* represent the most extreme life history, combining a vertebrate body plan with a characteristically invertebrate solution to survival in unpredictable conditions.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables, experimental procedures, and references and can be found with this article online at https://doi.org/10.1016/j.cub.2018.06.031.

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AUTHOR CONTRIBUTIONS

M.R. conceived and designed the study, M.V. and J.Ž. collected field data, M.P. performed histological analysis, and M.V. and M.R. drafted the manuscript.

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The cephalo-thoracic apparatus of *Caputoraptor elegans* may have been used to squeeze prey

Petr Kočárek[1,*]

Alienoptera is an insect order recently described from mid-Cretaceous amber [1] and is phylogenetically nested in the Dictyoptera lineage. Alienoptera currently comprises three species: *Alienopterus brachyelytrus* [1], *Alienopterella stigmatica* [2] and *Caputoraptor elegans* [3]. The most interesting is *Caputoraptor elegans*, which was recently described in *Current Biology* by Bai and colleagues [3] and which has an unusual cephalo-thoracic device formed by wing-like extensions of the genae and the corresponding edges of the pronotum. Bai and colleagues [3] suggested that the cephalo-thoracic apparatus may have been used to hold the female and male together during copulation. According to this possible function, the cephalo-thoracic apparatus of the female would fit together with the spread forewings of the male while the female was on the back of the male during copulation. This function was proposed based on examination of females and nymphs, and the authors stated that it could be falsified if a male with a similar apparatus were discovered. After examining a male nymph of this species (Figure 1), I here suggest that the cephalo-thoracic apparatus was not used for copulation but was instead used for predation and feeding.

The hypothesis that females use the cephalo-thoracic apparatus to hold onto males during copulation is improbable for two reasons. First, if the apparatus was used only for coupling, it would not be developed (or fully developed) in nymphs where it would not fulfil its purpose. Second, a character that is related to coupling and that occurs in only one of the sexes is almost always subject to sexual selection. Such selection, however, usually leads to sex-specific features in the sex with less investment in offspring, *i.e.*, in the sex that produces microgametes, and these are the males [4,5]. Because males are exposed to more intense sexual selection than females, conspicuous secondary sexual characters usually evolve in males rather than in females [6]. Although exceptions exist in insects [7], they are rare and related to cases in which male investment in offspring involves more than sperm donation, as is the case in Heteroptera: Belostomatidae [8]. Such a case, however, is unknown in dictyopteran insects [9,10].

As indicated above, the hypothesis about the use of the cephalo-thoracic device by the female seems unlikely due to the finding of a male nymph (Supplemental Information) with the fully developed apparatus. The studied nymph has nine visible abdominal coxosternites (Supplemental Information), and it is therefore an immature male. Bai and colleagues [3] also suggested that the cephalo-thoracic device may have been used for capturing prey or for defence.

A detailed study of the morphology of the cephalo-thoracic apparatus in the studied nymph (Figure 1; Supplemental Information) has revealed new findings that provides insight into its probable function. The area between the posterior part of the head (gula) and the anterior part of the pro- and mesothorax forms a cavity (Figure 1A). A sharp ridge surrounds the cavity, except in the area of the mouth and of the gap for the forelegs. The thoracic part of the ridge consists of projections of the pro- and mesonotum. The pronotal projections have a straight serrate distal part and a non-serrate proximal part, and these parts curve towards each other. A narrow, sharp extension of the mesonotum abuts the pronotal part and borders the gap for the forelegs.

If the cephalo-thoracic device is closed, the edge of the pronotal ridge is inserted beneath the edge of the wing-like extensions of the genae, and the non-serrate proximal part of the pronotal ridge fits into the narrow groove in the gula under a part of the gena and subgena. The short mesonotal ridge probably attaches to the distal part of the maxillae (stipes) in the closed stage. The closed cephalo-thoracic device remains opened only in the space formed partly

PAPER 3: Growth in the wild

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BRIEF COMMUNICATION

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Rapid growth and large body size in annual fish populations are compromised by density-dependent regulation

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Abstract

We tested the effect of population density on maximum body size in three sympatric species of annual killifishes *Nothobranchius* spp. from African ephemeral pools. We found a clear negative effect of population density on body size, limiting their capacity for extremely fast development and rapid growth. This suggests that density-dependent population regulation and the ephemeral character of their habitat impose contrasting selective pressures on the life history of annual killifishes.

KEYWORDS

Nothobranchius, plasticity, population regulation, somatic growth, sympatry, temporary habitat

Body size is a crucial phenotypic trait for individual survival and reproductive success (Blanckenhorn, 2000). In males, large body size indicates better condition, enables possession of superior territories and ultimately leads to larger male mating preference by females (Jennions *et al.*, 2001). In females, body size is positively associated with fecundity (Fleming, 1996; Monroe *et al.*, 2015) and in animals with asymptotic growth (such as fishes), fecundity increases disproportionately with body length (Barneche *et al.*, 2018). While larger body size and the ability to reach it quickly, is advantageous in many contexts, rapid growth is costly (Dmitriew, 2011). High investment into rapid growth is associated with late-life decreases in fecundity and survival (Hector & Nakagawa, 2012; Lee *et al.*, 2013).

Temporary habitats select for rapid growth (Dańko *et al.*, 2017). Temporary environments favour individuals that develop rapidly and reproduce before the conditions deteriorate. In addition, the long-term costs of rapid growth and development are not necessarily experienced in a temporary habitat, as environmental conditions often deteriorate before these costs are expressed.

Selection for large body size and rapid development interferes with the force of density-dependent regulation at the population level (Lorenzen & Enberg, 2002). A low population density with high availability of resources selects for individuals that rapidly exploit resources and consequently grow fast and have high reproductive success (fast life history). In contrast, high population density with intensive competition for resources favours individuals that utilise resources effectively despite growing more slowly and producing offspring at a lower rate (slow life history) (Reznick *et al.*, 2002). Thus, while at low population density selection for fast life history is intensified, high population density may impose constraints on rapid growth.

Killifishes (Cyprinodontiformes) from Africa and the neotropics have repeatedly evolved the ability to exploit ephemeral freshwater pools that develop during the rainy season (Furness *et al.*, 2015). Their life cycle is adapted to the transient nature of their habitat, with a rapid adult phase and long-term survival of desiccation-resistant eggs stored in the dry substratum. The length of the active life phase varies among species and regions, but is always constrained by annual desiccation and typically lasts <6 months (Reichard, 2015), with a high variability in the duration of the season (García *et al.*, 2018). Population densities vary greatly even among adjacent populations (Lanés *et al.*, 2016; Volcan *et al.*, 2019; Vrtílek *et al.*, 2018a) with embryonic survival success during the dry phase, or conditions in the previous wet phase as the potential drivers. Despite the causes of inter-population variation in density are largely unknown, density-dependent population effects strongly interact with selection for fast life history.

Here, we tested whether population density constrains rapid growth in annual killifishes. Using longitudinal data from six natural sites with African annual killifishes, we compared peak population density with maximum body size. We used the turquoise killifish, *Nothobranchius furzeri* Jubb 1971, a well-studied species in life-history

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evolution research (Cellerino *et al.*, 2016) and its coexisting sympatric species, *Nothobranchius orthonotus* (Peters, 1844) and *Nothobranchius pienaari* Shidlovskiy, Watters & Wildekamp, 2010 (Dorn *et al.*, 2014; Reichard *et al.*, 2009, 2017a). *Nothobranchius furzeri* exhibits particularly rapid life history. Fish hatch after the pools fill with rainwater (Polačik *et al.*, 2011) and grow rapidly to reach sexual maturity in as few as 2 weeks (Vrtílek *et al.*, 2018b), following a daily production of 20–120 eggs. The life history of the other two species is very similar (Blažek *et al.*, 2017; Vrtílek *et al.*, 2018a).

Nothobranchius spp. are income breeders with an immediate conversion of resources into current reproductive effort (Vrtílek & Reichard, 2015). They feed on a wide range of planktonic and benthic invertebrates whose availability severely decreases at high killifish density (Polačik *et al.*, 2014; Polačik & Reichard, 2010). Sexual dimorphism in colouration, morphology and body size is pronounced, with males being the larger sex. The growth of annual killifishes is particularly plastic and continues after sexual maturity when resources are available (Vrtílek & Reichard, 2015). Cross-sectional sampling of natural populations usually shows large body size differences among populations and relatively low intra-population variation (Vrtílek & Reichard, 2016), indirectly suggesting the effect of local conditions.

We measured total length (L_T; from tip of the mouth to the distal point of caudal fin) of *Nothobranchius* fish from six sites in southern Mozambique. The pools represented typical *Nothobranchius* habitats in the region and were visited over their entire adult-phase lifetime. Full details on the sampled sites are provided in Vrtílek *et al.* (2018a). The sites contained either only *N. furzeri* population (2 sites, Ch1 (22° 16′ 33″ S, 32° 53′ 54″ E) and LS5 (24° 24′ 59″ S, 32° 46′ 30″ E)), *N. furzeri* together with *N. orthonotus* (2 sites, Ch2 (22° 30′ 28″ S, 32° 34′ 54″ E) and LS2 (24° 18′ 15″ S, 32° 36′ 55″ E)) or *N. furzeri* with *N. orthonotus* and *N. pienaari* (2 sites, Ch3 (22° 33′ 17″ S, 32° 43′ 38″ E) and LS1 (24° 11′ 40″ S, 32° 22′ 45″ E)). Community complexity and species combinations were limited due to nested pattern of community assembly (Reichard *et al.*, 2017a). Fish were sampled during each visit of the site (3–6 visits per site, median interval 11 days; Figure 1) using a seine net (2.7 m long, 0.7 m deep, 4 mm mesh size). Standardised catch per unit effort (CPUE) consisted of five seine-net

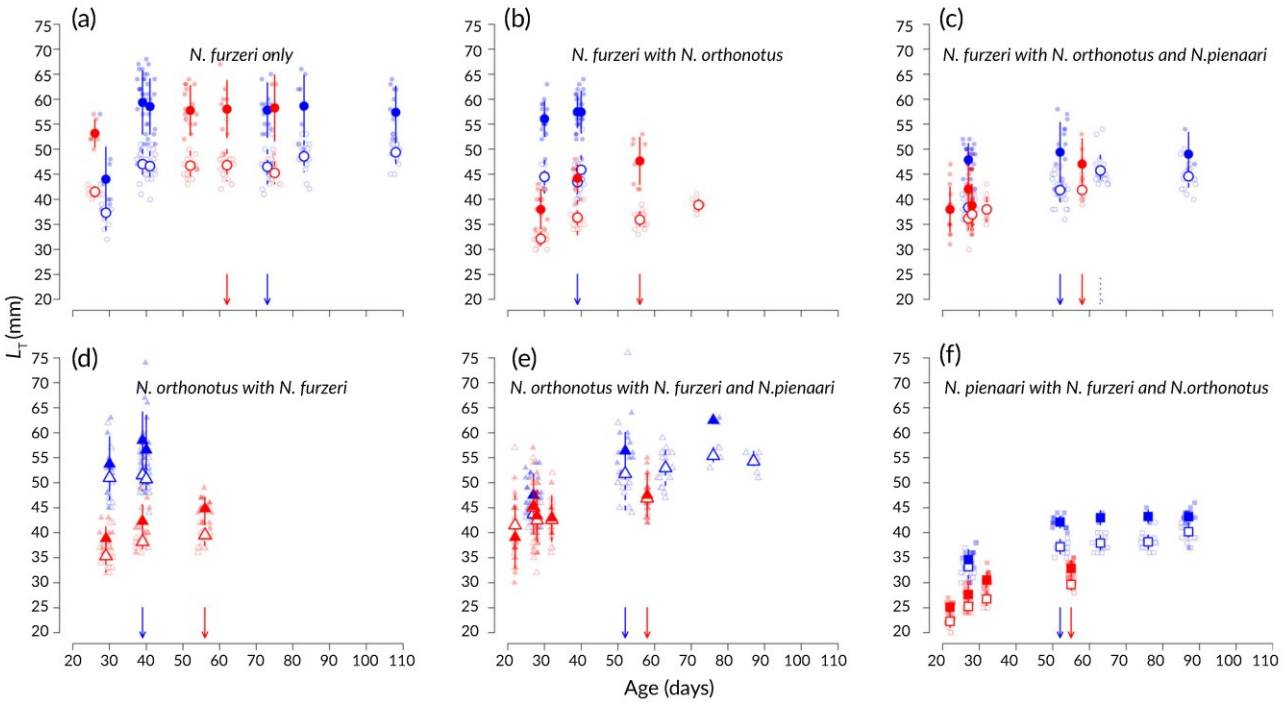


FIGURE 1 Seasonal dynamics in total length (L_T) structure in populations of three sympatric *Nothobranchius* species: (a)–(c) *N. furzeri* (—●—) Ch1 males, (--○--) Ch1 females, (—●—) LS5 males, and (--○--) LS5 females; (—●—) Ch3 males, (--○--) Ch3 females, (—●—) Ch2 males, and (--○--) Ch2 females; (—●—) LS2 males, (--○--) LS2 females, (—●—) LS1 males, and (--○--) LS1 females; (d), (e) *N. orthonotus* and (f): *N. pienaari*. Each panel shows a pair of populations with the same species community (*N. furzeri* only, *N. furzeri* with *N. orthonotus* or all three species) (—▲—) Ch3 males, (--△--) Ch3 females, (—▲—) Ch2 males, and (--△--) Ch2 females; (—▲—) LS2 males, (--△--) LS2 females, (—▲—) LS1 males, and (--△--) LS1 females; (—■—) LS2 males, (--□--) LS2 females, (—■—) LS1 males, and (--□--) LS1 females. Colour differentiates populations according to their maximum density: ■, the higher density population of the pair; ■, the lower density population of the pair. Small symbols represent measured values, large symbols indicate sample means (±SD), closed symbols and solid lines represent male values, open symbols with dashed lines are for females. ↓, The age when data for the analysis of the effect of population density on maximum body size were collected (Table 1, Figure 2). Location of populations: CH1, 22° 16′ 33″ S, 32° 53′ 54″ E; CH2, 22° 30′ 28″ S, 32° 34′ 54″ E; Ch3, 22° 33′ 17″ S, 32° 43′ 38″ E; LS1, 24° 11′ 40″ S, 32° 22′ 45″; LS2, 24° 18′ 15″ S, 32° 36′ 55″ E; LS5, 24° 24′ 59″ S, 32° 46′ 30″ E

hauls (5 m long) that covered all habitats in the pool. Fewer hauls were completed when the pool was too small to avoid repeated sampling of the same area. CPUE reflects population density because it directly assesses the number of individuals per unit area. A random sample of 15 individuals of each sex from each species was measured on-site on each occasion using a plastic sheet with a ruler. After measurements were taken, fish were released back into the pool.

Fish age was estimated from otoliths. *Nothobranchius* spp. hatch synchronously and the population is typically represented by a single age cohort (Polačik *et al.*, 2011; Reichard *et al.*, 2017b). During the first sampling at each site, we took a subsample of three individuals (mixed sex) per species per site, euthanized them in clove oil and stored them in 96% ethanol. Otoliths were dissected, polished and the number of daily increments was read by a commercial facility (Barcelona Otolith Reading Services, Spain; borsbarcelona@gmail.com). We estimated hatching date as the number of daily increments subtracted from the date of fish collection. To provide a population-level estimate of age, where different ages were recorded (maximum of 3 days difference), we used the earliest estimated hatching date (Vrtílek *et al.*, 2018a). Otolith samples were not available for site Ch3 so the hatching date was estimated by reading otoliths from the adjacent sites (Ch1 and Ch2). Hatching in *Nothobranchius* is triggered by pool inundation and we directly observed that these three pools were filled at the same time.

To test the effect of population density on maximum body length L_{Tmax}, we used values from the sampling dates when population-level L_T approached its maximum, corresponding to an age of 52–73 days in all pools except Ch3 (Figure 1). In Ch3 (with *N. furzeri* and *N. orthonotus*), the last sample was collected at the age of 39 days, just before the pool desiccated (Vrtílek *et al.*, 2018a). Given that Ch3 was a low-density site where we predicted larger L_T, any failure of fish to reach maximum L_T before habitat desiccation would have resulted in more conservative estimates in our analysis. Estimates of population density were based on maximum relative fish density (CPUE). Maximum CPUE was typically recorded during the first visit to the site (five of the six sites) and density steadily declined throughout the season (Vrtílek *et al.*, 2018a). The only exception was Ch3 where the pool desiccated earlier and relative population density increased (Vrtílek *et al.*, 2018a). Our use of maximum CPUE for population density estimates therefore corresponds to the period where it has the strongest effect on L_T as fish growth is most intense early in life (Quince *et al.*, 2008). Thus, CPUE represents a biologically relevant estimate, because lower growth early in life was not compensated by a longer growth period (Figure 1).

We fitted species-specific generalised least-squares models (GLS) with L_{Tmax} as the dependent variable and population density as a continuous explanatory variable in interaction with sex to test for different effects of population identity density on growth in females and males. We corrected for non-independence of the measurements from the same population by defining compound-symmetry correlation structure using population identity (Pekár & Brabec, 2016). We tested whether heteroscedasticity correction was necessary using the Akaike information criterion. To validate our results, we ranked populations according to their density. Fitting the models with ranked density resulted in a qualitatively equivalent outcome to the original analysis. We present the outcomes of the more biologically meaningful models with numeric density estimates. All analyses were performed using R software 3.5.1 (www.r-project.org) and package nlme 3.1-137 (Pinheiro *et al.*, 2018).

Population density was strongly negatively associated with L_{Tmax} in all species (Table 1 and Figure 2). Males were consistently larger than females (Table 1 and Figure 2), but the stronger effect of population density on male body size was pronounced only in *N. pienaari* (GLS: density × sex interaction: $F_{1,55} = 6.00$, $P < 0.05$). In *N. furzeri* ($F_{1,157} = 0.04$, $P > 0.05$) and *N. orthonotus* ($F_{1,110} = 0.03$, $P > 0.05$), no interaction between sex and population density was found.

In annual killifishes, selection for rapid growth and large body size is very strong in both sexes. Fish must reproduce before their habitat desiccates, resulting in sexual maturation in as few as 14 days (Vrtílek *et al.*, 2018b). Female fecundity is strongly correlated to body mass (Blažek *et al.*, 2017; Vrtílek & Reichard, 2015) while male hierarchy is contingent upon variation in body size (Polačik & Reichard, 2009). Here, we demonstrate that the selection for rapid growth and large body size imposed by habitat seasonality and reproductive strategy is severely compromised by density-dependent regulation at the population level.

Resource competition often governs density-dependent regulation and life-history evolution in fish (Bassar *et al.*, 2013). For example, high-density populations of Trinidadian guppies *Poecilia reticulata* Peters 1859 have evolved divergent life-history strategies from low-density populations. Stream sections that contain predatory fish sustain low-density *P. reticulata* populations, where survivors benefit from high *per capita* food availability compared with predator-free high-density populations (Bassar *et al.*, 2010). Predators thus indirectly select for a fast-living phenotype, expressed in rapid maturation and growth and high fecundity (Reznick, 1982). Under this scenario, natural selection favours individuals that are able to take advantage of the abundant resources associated with rapid growth (Sæther *et al.*, 2016).

In annual killifishes, population density does not appear to be regulated by predation, or at least, predation is not predictable. Various predators coexist with *Nothobranchius* spp., including piscivorous birds, lungfish and large predatory hemipterans, but with no geographic pattern (Reichard *et al.*, 2014). Instead, annual killifish population density is probably determined by particular local conditions that vary from year to year. Pool volume (a function of the amount of precipitation) and hatching success (based on the egg bank in the dry sediment) vary stochastically among years, limiting any directional density-dependent selection. Our long-term data suggest that population density varies considerably among years, at least in some populations (M.R., unpubl. data), making it implausible to suppose directional and stable selection pressure.

Life-history evolution in annual killifishes is contingent upon a strong aridity gradient, with a contrast in the duration of the aquatic phase between coastal wet and inland dry regions (Terzibasi Tozzini *et al.*, 2013). A previous experimental study contrasted populations of

	Estimate	SE	*t*-value[a]	*P*	ρ
N. furzeri (*n* = 161)					
Females	47.817	0.922	51.838	<0.001	0.064
Males	57.441	0.939	13.963	<0.001	
Density	−0.257	0.039	−6.557	<0.001	
N. orthonotus (*n* = 114)					
Females	56.472	0.827	68.317	<0.001	0.013
Males	60.394	0.881	5.734	<0.001	
Density	−0.372	0.028	−13.267	<0.001	
N. pienaari[b] (*n* = 59)					
Females	41.250	0.557	74.089	<0.001	0
Males	47.138	0.588	7.268	<0.001	
Density in females	−0.415	0.027	−15.206	<0.001	
Density in males	−0.095	0.039	−2.449	0.018[c]	

TABLE 1 Effect of population density on female and male asymptotic total length (L_T) in *Nothobranchius furzeri*, *N. orthonotus* and *N. pienaari*, where estimated values for females and males represent L_T at standardised (zero) density with one standard error (SE)

ρ, The value for the population intra-class correlation in each species' model (*i.e.*, the variance explained by population density divided by the total variance).
[a]Note that the *t*-values for males represent the contrast between the sexes, while *t*-values for females represent the statistical significance for their body size to be different from zero.
[b]Only two populations of *N. pienaari* were tested.
[c]The estimate and test statistic for the effect of density on male body size represents the difference from the effect of density in females, not the overall density effect in males.

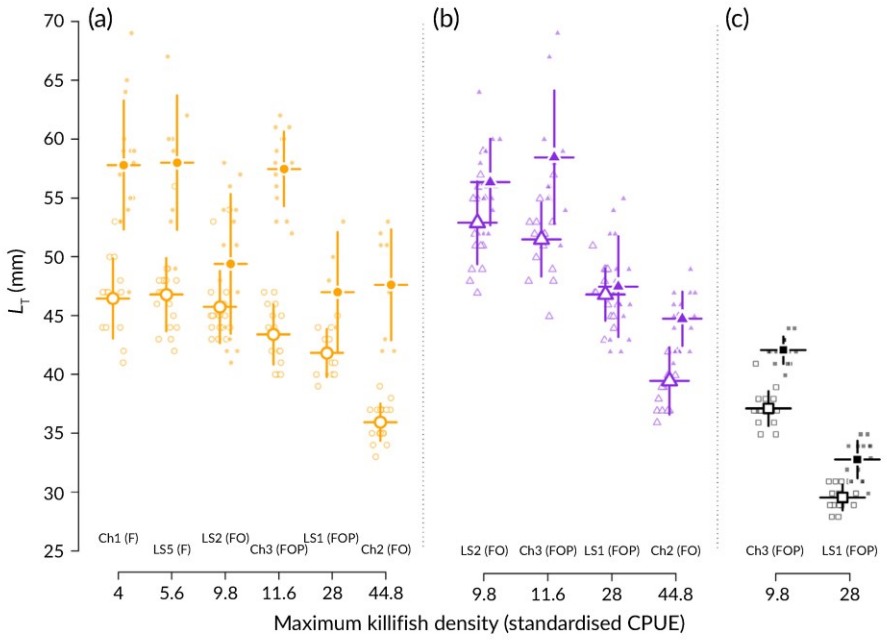


FIGURE 2 The effect of population density on female (open symbols) and male (closed symbols) total length (L_T) in (a) *Nothobranchius furzeri*, (–●–) males, and (–○–) Females (b) *Nothobranchius orthonotus* and (c) *Nothobranchius pienaari*. Large symbols, mean (±SD); small symbols, measured values. Species community structure is abbreviated next to the site names: F, *N. furzeri* only; FO, community of coexisting *N. furzeri* and *N. orthonotus*; FOP, community with all three species, *N. furzeri*, *N. orthonotus* and *N. pienaari*. *n.b.* The maximum recorded population density was analysed on a continuous scale but was ranked for graphical display. Location of populations: CH1, 22° 16′ 33″ S, 32° 53′ 54″ E; CH2, 22° 30′ 28″ S, 32° 34′ 54″ E; Ch3, 22° 33′ 17″ S, 32° 43′ 38″ E; LS1, 24° 11′ 40″ S, 32° 22′ 45″; LS2, 24° 18′ 15″ S, 32° 36′ 55″ E; LS5, 24° 24′ 59″ S, 32° 46′ 30″ E

annual fishes from dry and wet regions in a common laboratory environment and predicted more rapid life history in populations from dry region. However, no difference in the rate of juvenile growth, sexual maturation or maximum size was found between dry- and wet region populations, despite corroboration of the predicted difference in lifespan and functional aging rate (Blažek *et al.*, 2017). Here, we show that realised growth rate in natural populations is strongly contingent upon local population density, apparently overriding potential directional selection for rapid development across environmental gradient.

Rapid development and early maturation are predicted to impair further somatic growth (Quince *et al.*, 2008). In the present study, fish exhibited all the external signs of sexual maturity from the first sampling but sustained growth throughout the monitoring period. In the wild, juvenile growth of *Nothobranchius* spp. was much greater (at least 35 mm at the age of 30 days even in high density populations of *N. furzeri*) than in the laboratory (L_S; standard length of 20 mm at the age of 30 days (Vrtílek & Reichard, 2015) and L_T 30 mm at maturity after 32 days (Blažek *et al.*, 2017)). In the laboratory, the growth

rate of adult fish rapidly adjusted to the amount of food at the age of 2 months and individuals were capable of rapid compensatory growth (Vrtílek & Reichard, 2015). The present study suggests that the capacity for compensatory growth pertains to natural populations. Adult mean L_T increased by approximately 30% since maturity in both sexes in all three species, as population density decreased steadily over the course of the season (Vrtílek *et al.*, 2018a). The sustained growth was especially apparent in fish from higher density populations.

In addition to population density, community complexity may affect fish growth (Fox, 1994). We acknowledge that the distribution of population densities in our study was entirely collinear with community complexity, with the highest population densities in the most complex killifish communities (three coexisting species) and the lowest population densities in single-species communities. Whether there is a functional link at the habitat level between sustenance of dense populations and rich communities is unclear (Reichard *et al.*, 2017a), but examining the species-specific slopes of the density effect on the maximum body size suggests that *N. furzeri* (present in all communities) is the species with growth rate least affected by population density while *N. pienaari* was the most affected and inhabited exclusively the most complex communities. This link has to be taken with extreme caution due to the limited number of replicated communities, though it may provide an explanation for annual killifish community assembly and the relative rareness of certain species. While we have not studied the mechanistic causes of density-dependent growth regulation, we note that *Nothobranchius* killifish are aggressive. Increased aggression and the associated stress response may have contributed to growth limitation in addition to the indirect effect of competition for food.

Ephemeral habitats impose strong selection for rapid development (Dańko *et al.*, 2017) but directional selection is rarely apparent in the wild because of variation in local conditions and phenotypic plasticity (Pujol *et al.*, 2018). Realised somatic growth in temporary habitats is regulated by population density while the evolution of growth rate is mainly shaped by trade-offs between current and future reproductive effort. Indeed, annual killifishes exhibit great phenotypic plasticity (Cellerino *et al.*, 2016), adjusting their growth to the prevailing ecological conditions both in the wild and the laboratory with very limited evidence so far for local adaptation.

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AUTHOR CONTRIBUTIONS

M.R. conceived and designed the study, M.V., J.Ž., M.R., M.P. and R.B. collected the data, M.V. analysed data and drafted manuscript, all authors revised and approved the final version of the manuscript.

ETHICAL STATEMENT

All fieldwork was carried out in accordance with legal regulations of Mozambique (collection licence: ADNAP-170/7.10/16). The research procedures were approved by the ethical committee of the Institute of Vertebrate Biology, Czech Academy of Sciences.

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PAPER 4: Monitoring demography of wild populations

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Longitudinal demographic study of wild populations of African annual killifish

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The natural history of model organisms is often overlooked despite its importance to correctly interpret the outcome of laboratory studies. Ageing is particularly understudied in natural populations. To address this gap, we present lifetime demographic data from wild populations of an annual species, the turquoise killifish, *Nothobranchius furzeri*, a model species in ageing research, and two other species of coexisting annual killifishes. Annual killifish hatch synchronously, have non-overlapping generations, and reproduce daily after reaching sexual maturity. Data from 13 isolated savanna pools in southern Mozambique demonstrate that the pools supporting killifish populations desiccated 1–4 months after their filling, though some pools persisted longer. Declines in population size over the season were stronger than predicted, because they exceeded the effect of steady habitat shrinking on population density that, contrary to the prediction, decreased. Populations of *N. furzeri* also became more female-biased with progressing season suggesting that males had lower survival. *Nothobranchius* community composition did not significantly vary across the season. Our data clearly demonstrate that natural populations of *N. furzeri* and its congeners suffer strong mortality throughout their lives, with apparent selective disappearance (condition-dependent mortality) at the individual level. This represents selective force that can shape the evolution of lifespan, and its variation across populations, beyond the effects of the gradient in habitat persistence.

Model species provide key insights into many aspects of fundamental and applied biological research. However, despite the fact that laboratory conditions often differ considerably from model species' natural environment, it is usual to interpret the outcome of laboratory studies without the context of the evolutionary and natural history of these species. This is unfortunate because information about the selective forces (environmental or biotic) driving particular adaptations may provide stronger insights into the general understanding of the key findings in laboratory populations[1].

For a long time, the mere existence of ageing in the wild was regarded negligible and unimportant. Animals were generally considered to succumb to predation or pathogens before they started to age. However, recent evidence demonstrates that ageing is widespread in natural populations[2]. Despite the challenges involved in studying ageing in natural populations, the understanding of the ageing process (demographic, functional and reproductive declines) gained from natural populations is indispensable[3–7]. For example, Warner *et al.*[6] challenged the classic view by showing that even a long-lived ectotherm (painted turtle, *Chrysemys picta*) can suffer from considerable fitness reduction and elevated mortality in advanced age. To that end, describing the dynamics of demographic parameters in wild populations is crucial to our understanding of how natural selection acts on life-history evolution.

The African turquoise killifish, *Nothobranchius furzeri*, is an established model in several biological disciplines[8,9], particularly in ageing research[10–12]. *Nothobranchius furzeri* is the shortest-lived vertebrate that can be bred in captivity, with a median lifespan of 2–6 months[9,13], maximum lifespan of approximately 1 year[14] and laboratory generation time of 2–4 months[9,15]. These figures are more similar to the life-history schedule of invertebrate models than for a vertebrate[8]. Despite its extremely short lifespan, *N. furzeri* possesses all the hallmarks of vertebrate ageing and its lifespan is condensed rather than terminated prematurely[8]. Over the last decade, exceptional insights have been gained into the biology of ageing in *N. furzeri* and the species is at the forefront of the current fundamental and applied ageing research agenda[8,10–12,16].

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Figure 1. Male and female of turquoise killifish, *Nothobranchius furzeri*.

In contrast, very little is known about the ageing of *N. furzeri* in natural populations. The lifespan and ageing of *N. furzeri* in natural populations have never been estimated and are subject to scientific debate. While Terzibasi *et al.*[17] suggested that mortality in wild populations is primarily caused by habitat desiccation, Reichard *et al.*[18] demonstrated that it is strongly male-biased and hence likely includes components of sex- and condition-dependence. This is an important difference because theory suggests that the evolution of lifespan and ageing is strongly contingent upon the source of mortality[19]. When mortality is condition-independent (as in the case of habitat desiccation), increased risk of mortality is predicted to select for shorter lifespan and more rapid ageing[20,21]. However, when mortality is condition-dependent and low condition individuals disappear from the population earlier, the predictions regarding selection for lifespan and ageing are more complex[22–24]. Strong condition-dependent mortality could select for high individual condition overall (e.g. large body size, high immunity or social dominance), ultimately leading to extended lifespan and slower ageing in environments where populations are protected from the source of condition-dependent mortality, such as in captivity[25–27].

In this study, we focus on the demographic parameters of *N. furzeri* populations and their sympatric congeners in the wild. Demographic (actuarial) ageing describes the increase in risk of mortality with increasing age at the population level. A better understanding of the dynamics of mortality in natural conditions will help to identify underlying relationships and selective forces that have led to the evolution of an extremely short lifespan in *N. furzeri* and thus provide data essential for the interpretation of laboratory-based studies.

Nothobranchius furzeri, as with all other *Nothobranchius* species, are adapted to temporary pools that are formed during seasonal rains and desiccate early in the dry season[28]. Fish hatch from the eggs that survive the dry season in diapause, forming a single age cohort that inhabits the pool until its desiccation[29,30]. Dispersal among the individual pools is limited to years with exceptionally high precipitation and populations are strictly spatially separated in most generations[28,31,32]. In the laboratory, *N. furzeri* grow rapidly and mature in as little as 3 weeks under favourable conditions[15]. The sexes are markedly dimorphic with brightly coloured males and inconspicuous brown females (Fig. 1). Their sex-determination system is genetic, but sex chromosomes are not present[33]. The sex ratio is equal at the time of sexual maturity but many natural populations have female-biased adult sex ratios, likely a consequence of sexual selection (male-biased predation, higher male frailty or male-male competition)[18]. This indicates that while the ultimate disappearance of populations is caused by pool desiccation[14,17], *N. furzeri* are subject to significant mortality throughout their lives. Identifying the sources of *Nothobranchius* spp. mortality in natural populations should provide critical insight for our understanding of the complex evolution of ageing.

Here, we present a longitudinal demographic study of wild populations of *N. furzeri* and their two sympatric congeners, *N. pienaari* and *N. orthonotus*. We followed numerous populations in southern Mozambique over a five-month period and examined the temporal dynamics in habitat conditions and killifish demographic (abundance, density, sex ratio) and community (species ratio) parameters. We predicted that population abundance would decline over the season while population density would increase due to the reduction in pool area and volume. We also predicted more rapid disappearance of males from the populations, leading to a female-biased sex ratio. Finally, we predicted a faster decline in the populations of *N. furzeri* compared to two sympatric *Nothobranchius* species, in accordance with the differences in their lifespans in a laboratory environment.

Results

Overview. We collected data for a total of 13 sites with up to three *Nothobranchius* species present (Table 1). Nine of those sites provided long-term demographic data. At two sites (LS3, LS4), the pools desiccated soon after the fish matured. One site (LS6) exhibited extremely low abundance of fish and another site (LN3) was too deep to support quantitative sampling. These 4 sites were used only in some of the planned analyses (for details see Table 1).

Hatching date estimates. We estimated hatching date from readings of daily otolith increments for 10 sites (3 specimens per species per site) (Supplementary Table 1). There were three main periods of hatching across the study area, generally contingent upon division into three phylogeographic clades of *N. furzeri* (Table 1, Fig. 2). Fish from the Limpopo South and Chefu clades hatched in early and late January, respectively. The populations

Site	GPS coordinates	Species present	F clade	Hatching date	CPUE visits	Last CPUE	Final age	Analyses
Ch1	E 32°53′54″, S 22°16′33″	F, O	Chefu	26.1.16	13	25.5.16	120+	population size, density, sex ratio
Ch2	E 32°34′54″, S 22°30′28″	F, O, P	Chefu	26.1.16	1	11.3.16	72/−	sex ratio, species ratio
Ch3	E 32°43′38″, S 22°33′17″	F, O	Chefu	26.1.16*	3	5.3.16	45/−	population size, density, sex ratio, species ratio
LN1	E 32°34′01″, S 23°28′24″	F, O, P	Limpopo North	12.3.16	5	25.4.16	51/−	population size, density, sex ratio, species ratio
LN2	E 32°34′21″, S 23°30′14″	F, O	Limpopo North	12.3.16	—	NA	71/−	population size, sex ratio, species ratio
LN3	E 32°34′40″, S 23°31′47″	F, O	Limpopo North	10.3.16	—	NA	77+	sex ratio
LN4	E 32°36′01″, S 23°38′44″	F, O, P	Limpopo North	15.3.16	10	12.5.16	58/−	population size, density, sex ratio
LS1	E 32°22′45″, S 24°11′40″	F, O, P	Limpopo South	18.3.16	10	9.5.16	59/−	population size, density, sex ratio, species ratio
LS2	E 32°36′55″, S 24°18′15″	F, O, P	Limpopo South	10.1.16	5	21.4.16	110/−	population size, density, sex ratio, species ratio
LS3	E 32°44′33″, S 24°19′32″	F	Limpopo South	10.1.16*	—	NA	33/−	population size
LS4	E 32°41′37″, S 24°19′37″	F	Limpopo South	10.1.16*	—	NA	33/−	population size
LS5	E 32°46′30″, S 24°24′59″	F, P	Limpopo South	11.1.16	2	26.3.16	86/o	density, sex ratio
LS6	E 32°46′44″, S 24°25′08″	F, P	Limpopo South	10.1.16	—	NA	63/−	species ratio

Table 1. Summary table for data obtained from the study sites. The abbreviation for *Nothobranchius* species present are F for *N. furzeri*, O for *N. orthonotus* and P for *N. pienaari*. F clade is phylogeographic lineage of *N. furzeri*[31]. Hatching date was estimated based on otolith readings and for sites, where not collected (*), the hatching date was estimated based on the hatching date estimates for fish in nearby sites (Supplementary Table 1). Final age is number of days the fish reached during our monitoring. The fish were present until pool desiccation (/−), fish disappeared, while the pool was still present (/o), or fish survived longer than the sites were monitored (+) (see also Fig. 2).

from Limpopo North hatched only after heavy late-season rains in mid March. The late-season hatching in site LS1 (Limpopo South) also corresponded with the rains in mid March.

Population size and population density dynamics. The size of study pools was greatest immediately after filling and gradually decreased throughout the study period (Fig. 2). All but two pools desiccated before the end of our sampling (Fig. 2). The duration of existence of individual pools ranged from 33–110 days. One pool (site Ch1) persisted for more than 120 days (with *Nothobranchius* spp. present) when last visited on 25 May, as the fieldwork period was concluded. Another pool that still contained fish on 26 May (site LN3) had existed for 77 days at that time, as it was filled late in the rainy season.

Population size was estimated at 9 sites using either Catch-Per-Unit-Area (CPUA) or Capture-Mark-Recapture (CMR) estimates. Population estimates ranged over three orders of magnitude – from 53 individuals (*N. orthonotus* at LN2) to almost 20,000 individuals (*N. furzeri* at LS3) (Table 2). Repeated population size estimates, completed for two *N. furzeri* populations (LS2 and LS5), demonstrated that fish abundance declined sharply within a single month. A major decline to 14% of the original population size (LS5) was associated with a reduction of pool surface area to 42% of its original extent. The population decline to 47% of the original population size in the pool LS2 was accompanied by a reduction to 26% of pool surface area measured during the first CMR estimate (Table 2).

Fish density was estimated as standardized Catch-Per-Unit-Effort (CPUE, the number of individual fish per net haul) at relatively regular intervals throughout the study period. An increase in fish density during the season was recorded only at a single site (Ch3), despite our prediction that density would increase as pool volume gradually shrank. Instead, fish density decreased significantly throughout the season in 3 out of 7 regularly sampled pools (3–13 visits per site) while no significant trend was detected in the remaining 3 pools (Fig. 3, Supplementary Table 2). Further, in two populations where CPUE indicated no decline in population density (CPUE), CMR sampling did detect a decrease (Table 2). Using the same approach, we additionally analysed density of *N. furzeri* separately from the other two species; the trends were comparable though weaker, as this analysis was based on a subset of data on all three species combined (Supplementary Fig. 1).

We were also unable to collect any *Nothobranchius* spp. despite substantial effort at one of the monitored, relatively small-sized, sites (LS5) 1 month before the pool actually desiccated (Table 1, Fig. 2). Collectively, this suggests that significant mortality is widespread among *Nothobranchius* spp. populations long before the pool desiccates.

Site	Species	Method	Capture date	Area m^2	Age	Captured fish	Recapture probability	Population size estimate (lower CI; upper CI)
Ch1	F	CMR-e	7.3.16	2138	41	145	0.19	748 (515; 981)
LN1	F	CMR-e	9.4.16	255	28	192	0.60	318 (264; 372)
LN2	F	CMR-e	19.4.16	1518	38	237	0.52	457 (402; 512)
—	O	CMR-e	19.4.16	1518	38	28	0.52	53 (31; 75)
LN4	F	CMR-e	10.4.16	626	26	210	0.15	1422 (1103; 1741)
LS1	F	CMR-e	11.4.16	836	24	74	0.36	204 (45; 363)
—	O	CMR-e	11.4.16	836	24	133	0.52	256 (189; 323)
LS2	F	CMR-f	5.2.16	3150	26	191	0.18	1054 (694; 1414)
—	—	—	2.3.16	812	52	41	0.08	492 (184; 800)
—	O	CMR-f	2.3.16	812	52	47	0.15	315 (135; 495)
—	P	CMR-f	2.3.16	812	52	126	0.11	1099 (694; 1504)
LS3	F	CPUA	23.1.16	356	13	877	0.34–0.50#	8714–19263~
LS4	F	CPUA	24.1.16	108	14	128	0.48–0.75#	577–1120~
LS5	F	CMR-f	6.2.16	2550	26	414	0.10	4247 (2827; 5667)
—	—	—	2.3.16	1081	51	140	0.23	613 (427; 799)

Table 2. Population-size estimates from Capture-Mark-Recapture (CMR) or Capture-per-Unit-Area depletion (CPUA) method. In the CMR, adult fish were marked with either visible implant elastomer tags (CMR-e) or by fin-clipping (CMR-f). The population size in subadult fish was estimated only using enclosures and CPUA. The ranges of capture probability (#) and population size estimate (~) for CPUA are given.

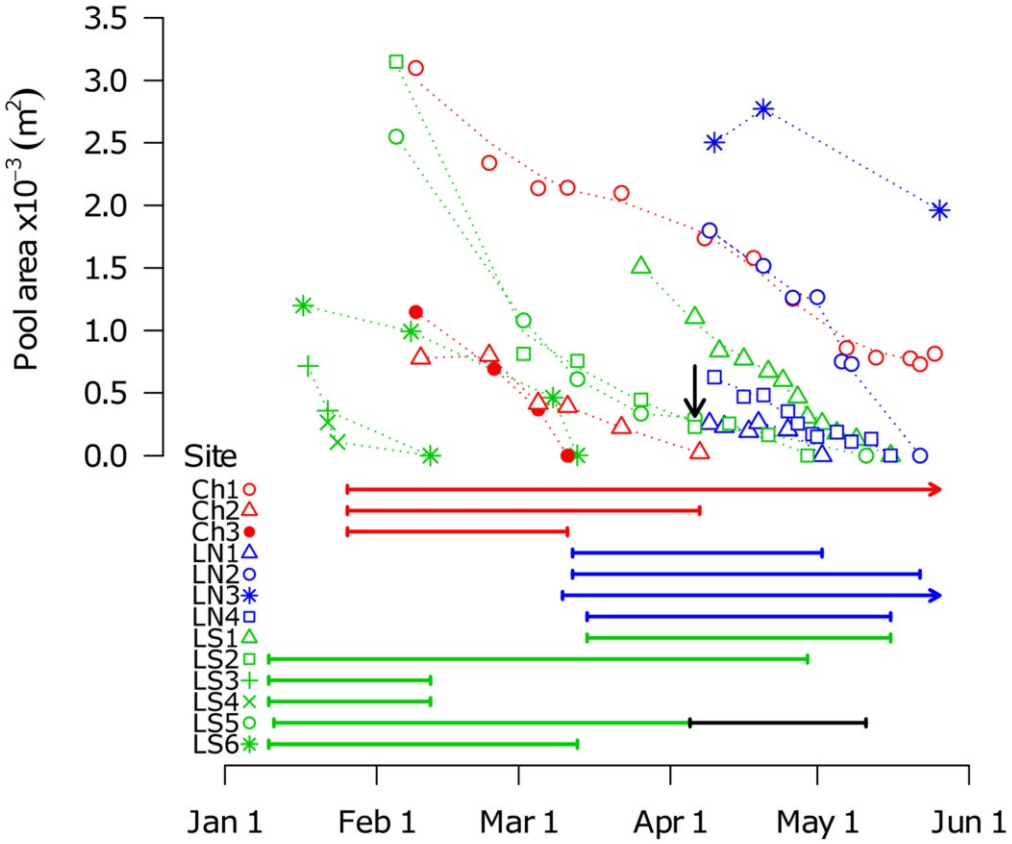


Figure 2. Pool area of the study sites throughout the 2016 rainy season. The points denote pool area when visited and the smooth dashed lines are fitted using LOESS function. The zero pool area marks the date when the pool was found to be dry. The black arrow shows a moment when fish disappeared from the pool LS5, while water was still present. The site-specific full lines show period between hatching and either pool desiccation, fish disappearance (site LS5, with the black line for period without fish), or termination of the fieldwork (arrowhead).

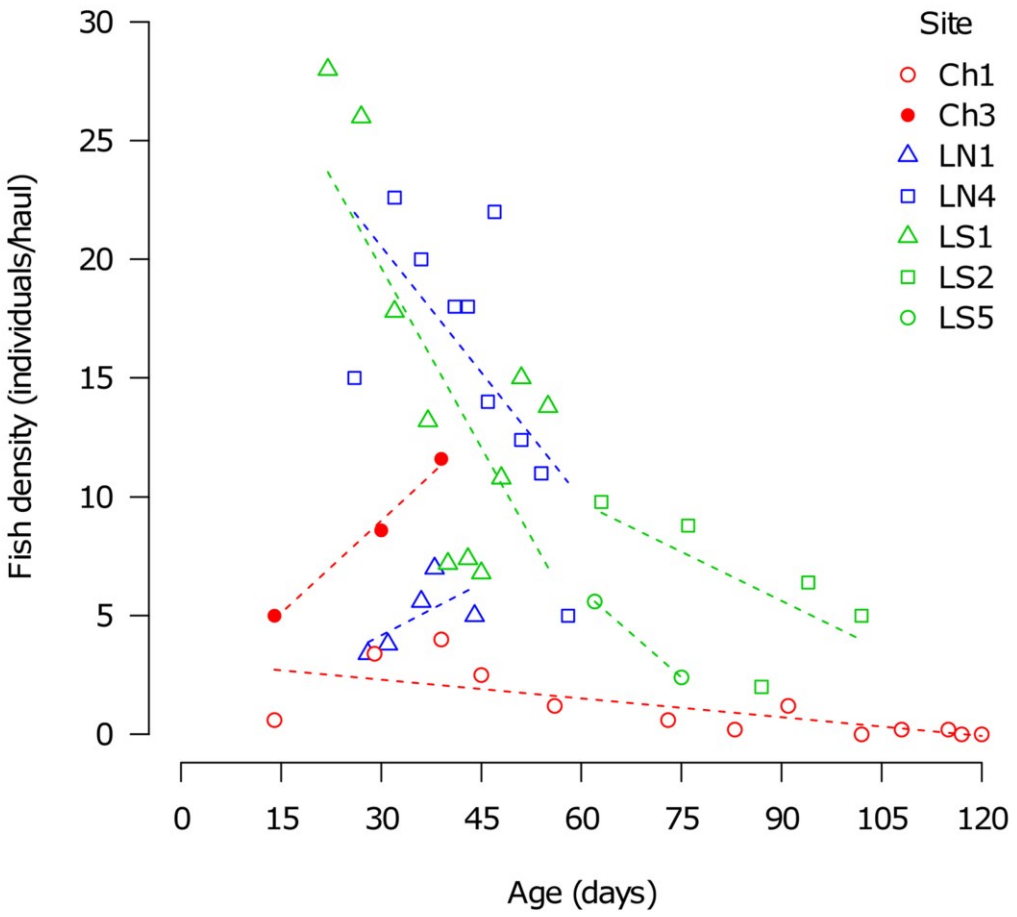


Figure 3. Temporal pattern of fish density at the study sites. Only sites with at least 3 visits with CPUE performed were analysed. The lines fitted represent predicted trend for each site from the generalised linear squares model (the estimated slope coefficients including their 95% CI are given in Supplementary Table 2).

Seasonal dynamics in the adult sex ratio. We examined temporal dynamics in sex ratio as the proportion of males from the 'Total catch' (CPUE sampling complemented with additional sampling effort) in the three study species – *N. furzeri* (10 monitored populations, 70 sampling events, 7376 captured individuals), *N. orthonotus* (5 populations, 30 samplings, 1529 individuals) and *N. pienaari* (2 populations, 18 samplings, 1627 individuals). The seasonal trend in sex ratio varied among species (Fig. 4). The relative proportion of males declined in *N. furzeri* populations (GLMM: Estimate = −0.015, SE = 0.005, z-value = −3.190, P = 0.001, Fig. 4a). High interpopulation variability resulted in no overall trend in *N. orthonotus* (GLMM: Estimate = −0.006, SE = 0.006, z-value = −1.036, P = 0.300, Fig. 4b). The proportion of male *N. pienaari*, unexpectedly, increased throughout the season in both populations (GLMM: Estimate = 0.010, SE = 0.004, z-value = 2.221, P = 0.026, Fig. 4c), though a numerical male-bias was recorded only in one population (Fig. 4c).

Species ratio. We performed 7 samplings at two sites where *N. furzeri* was sympatric with *N. orthonotus*, 5 samplings at a site where *N. furzeri* coexisted with *N. pienaari* (LS6), and 33 in four pools where all three species co-occurred. We did not record any non-annual teleost fish. In total, we collected 2476 individual *N. furzeri*, 1620 *N. orthonotus* and 1842 *N. pienaari*. The relative proportion of the three sympatric *Nothobranchius* species was site-specific (Fig. 5). *Nothobranchius furzeri* was present in all monitored pools, but its proportion in the community varied across pools. Neither the temporal pattern in abundance of *N. furzeri* (GLMM: effect of sympatry with *N. orthonotus* only – Estimate = 0.003, SE = 0.014, z-value = 0.198, P = 0.843; effect of sympatry with *N. pienaari* only – Estimate = −0.014, SE = 0.016, z-value = −0.860, P = 0.390; sympatry with both *N. orthonotus* and *N. pienaari* was used as an intercept), nor its relative abundance was affected by the presence or absence of particular coexisting species (GLMM: effect of sympatry with *N. orthonotus* only – Estimate = 0.707, SE = 0.386, z-value = 1.830, P = 0.067; effect of sympatry with *N. pienaari* only – Estimate = 0.430, SE = 0.550, z-value = 0.781, P = 0.435; sympatry with both *N. orthonotus* and *N. pienaari* was used as an intercept) and there was no overall seasonal trend in the proportion of *N. furzeri* in the *Nothobranchius* spp. community over the season (GLMM: Estimate = −0.003, SE = 0.005, z-value = −0.541, P = 0.589). There was no consistent pattern of temporal dynamics in species ratio and the sites were relatively stable in their community structure (Fig. 5).

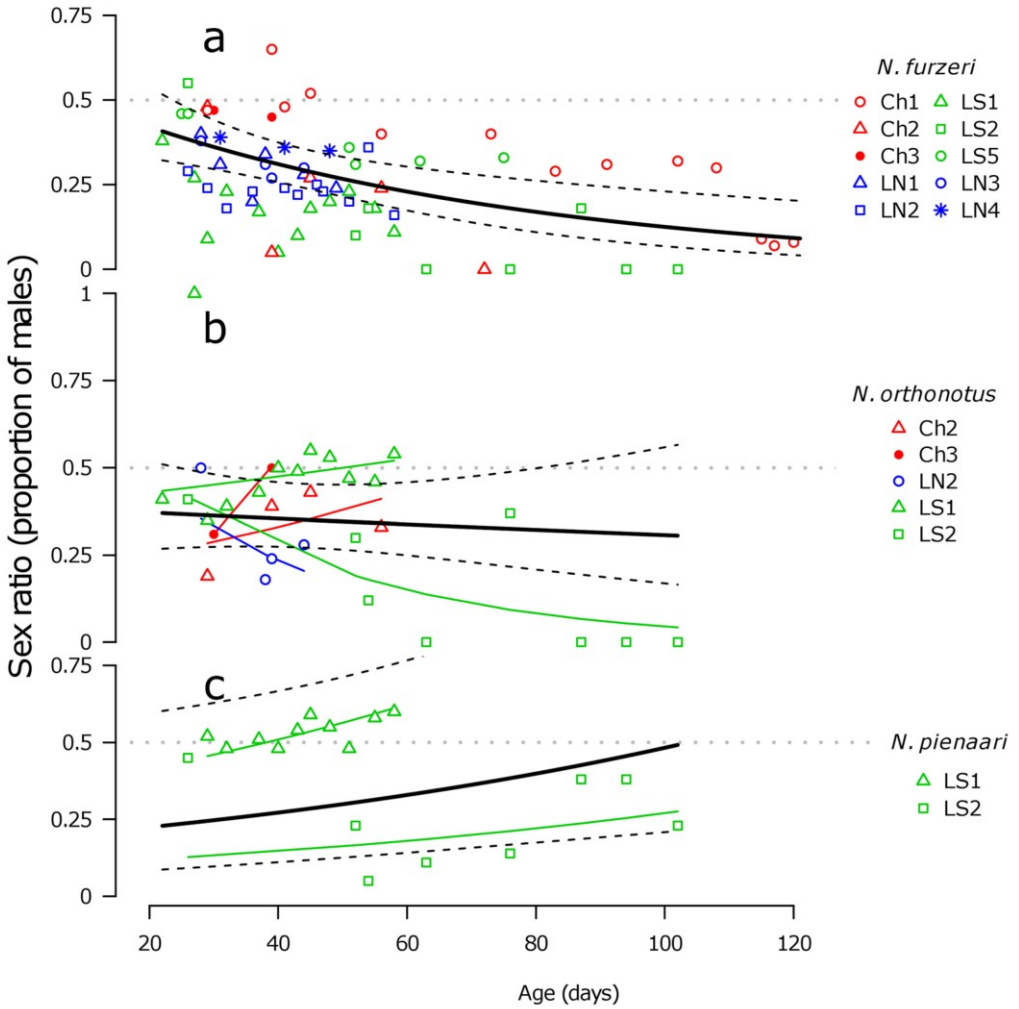


Figure 4. Temporal change in sex ratio. Seasonal change in sex ratio (proportion of males) in the three studied *Nothobranchius* species. In *N. furzeri* (**a**), the overall trend is negative, in *N. orthonotus* (**b**) inconclusive (population-specific) and in the two populations of *N. pienaari* (**c**), the proportion of males increased throughout the season. The thick black line gives overall trend estimated by the GLMM and dashed lines denote 95% CI.

Discussion

The pools supporting killifish populations typically desiccated 1 to 4 months after filling but killifish sometimes disappeared from the pool well before the terminal habitat deterioration. We demonstrated that the density and abundance of *Nothobranchius* spp. populations declined considerably during the course of the season (Fig. 3, Table 2). This decline in population density contrasted with predicted increase in the population density due to gradual habitat shrinkage. Population size was in the order of hundreds to thousands of young adults (3.5–7.5 weeks old) but estimated to be up to an order of magnitude larger in subadults (2 weeks old fish). The sexes were subject to differential mortality but the seasonal dynamic in sex ratio was species-specific (Fig. 4). While male mortality was higher than female mortality in *N. furzeri*, sex bias in mortality was population-specific in *N. orthonotus*. In *N. pienaari*, the initial female bias was buffered later in the season. The three sympatric species co-existed at locally variable, but temporally relatively stable, proportions and *N. furzeri*, the shortest living species, did not disappear from the pools any earlier than the other two species.

The duration of the aquatic phase varied among pools, but with no relation to geographic location of the site (Fig. 2, Table 1). A previous study analysed data from visits to sites over multiple seasons (2011–2015) and demonstrated that pools in the drier part of the study area (Chefu region, Supplementary Fig. 2) desiccated significantly sooner than pools in the more humid Limpopo North region[14]. This outcome was consistently confirmed using independent datasets obtained from datalogger readings that detected desiccation as an abrupt change in ambient temperature amplitude[17,30]. In 2016, when the current study was conducted, there was a particularly light rainy season without the annual monsoon precipitation and intensive rainfall was regionally localised. We hypothesize that this masked the pronounced regional differences in the timing of pool desiccation observed in

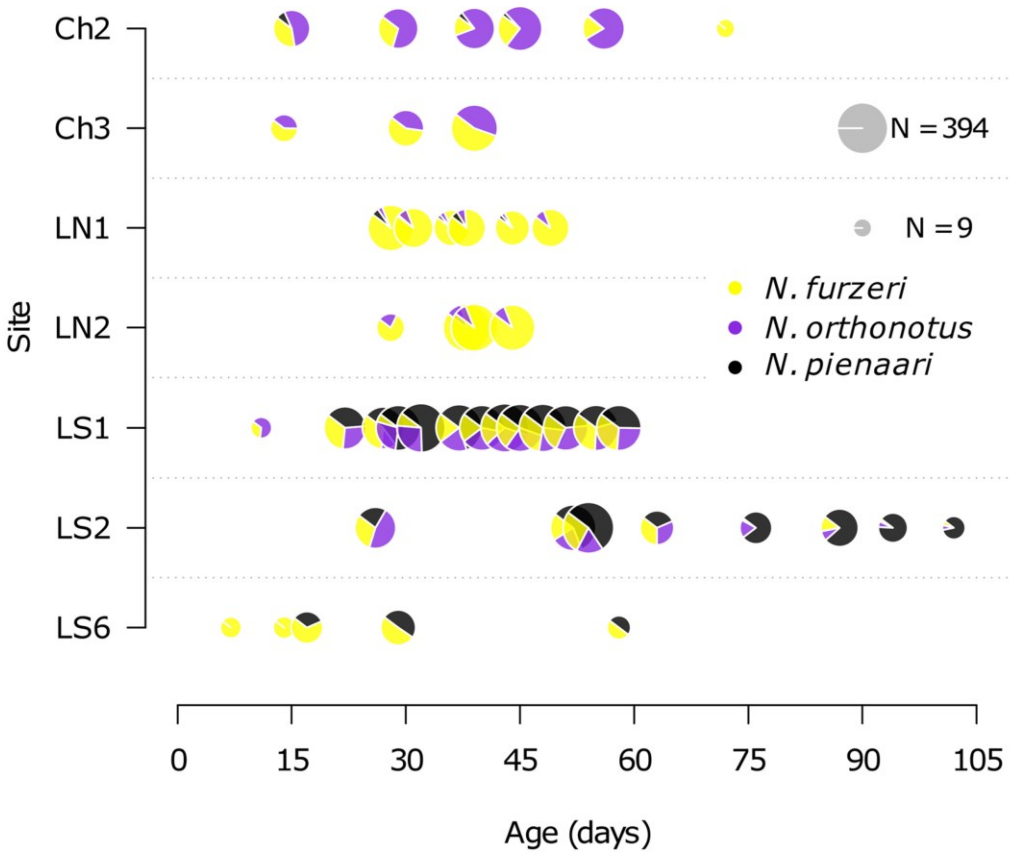


Figure 5. Proportions of up to 3 sympatric *Nothobranchius* species at 7 study sites throughout the season. The size of a pie gives number of fish in Total catch at each visit, thereby not necessarily showing population abundance. Note that only sites with 10 individuals captured per species at least once are included.

previous years. Nevertheless, our data demonstrate that the duration of the aquatic phase is variable among pools. Some populations survived only 33 days after hatching and almost half of the study sites (6 of 13) desiccated less than two months after filling (Table 1). In those pools, the main source of adult mortality was habitat desiccation. In contrast, study populations that persisted longer declined strongly before the pool desiccated, indicating that other factors, such as individual frailty to environmental and biotic challenges (e.g. high ambient temperature and low oxygen levels or aggressive encounters) or predation, were responsible for major mortality. Individuals from those populations were hence arguably subject to significant condition-dependent mortality (i.e. selective disappearance). Given that there is interannual variability in duration of specific pools[28], the populations experience variable strength of random (pool disappearance) and selective (seasonal decline) mortality over successive generations[8]. This makes the selection pressure on annual killifish life history temporally dynamic, providing a potential explanation of the mismatch between selection on ageing and on other life history traits identified in an experimental study[14]. Under standardised laboratory conditions, lifespan and ageing varied predictably between populations from dry and wet regions, while reproductive allocation and behavioural and physiological aspects of the pace-of-life syndrome did not differ between contrasting populations[14].

The limit imposed by pool existence is the ultimate selection force shaping annual killifish life history that is manifested by fast growth, early sexual maturation, high investment into reproduction and rapid ageing[8,14,15]. Naturally, a longer habitat duration provides more scope for condition-dependent mortality factors, as illustrated in Fig. 4 in ref.[8]. For example, both predation rate and predator richness are higher in temporary water bodies with extended duration[34,35]. High population density is known to facilitate the effect of condition-dependent factors on survival across various taxa[36–38]. Density-dependent regulation in *Nothobranchius* spp. may be further accentuated due to high levels of male aggression[14,39] and their high *per capita* resource requirements[15,40]. We found support for this hypothesis as *Nothobranchius* spp. populations with high densities displayed the steepest density decreases (Fig. 3).

Individual marking and frequent sampling may potentially increase mortality of repeatedly sampled fish. We are confident that that our sampling regime did not cause significant mortality. We addressed this issue seriously at the stage of designing our sampling protocol as well as during the fieldwork. We only used a short distance haul (5 m) with the seine net to minimize negative impact of longer-term disturbance from net dragging. All collected fish were placed in a bucket with fresh water and strong aeration if not released immediately after capture. Both

marking methods (finclipping, elastomer injection) have low impact on fish survival, as proven in previous pilot and published studies[14,18]. During elastomer tagging, that was more invasive marking technique and included anaesthesia, mortality of 0.5–2.5% was recorded. Finally, we documented no immediate mortality (presence of death fish) after returning the sampled fish back to the pool, and no delayed mortality after visit of the pool few hours after sampling (typically next morning).

The estimates of adult population size were an order of magnitude lower than for subadults. Population abundance for juveniles of 2 weeks of age was estimated as between 500 and 20,000 individuals using repeated depletions of enclosed sampling areas (CPUA estimate). The maximum estimated adult population size was always <5,000 individuals, using CMR methods. The use of different methods was imposed by logistic constraints; small juvenile fish could not be marked without compromising their condition and survival, while adult fish could not be enclosed in a netted area without a bias arising from disturbance and escape reaction. In fishes, juvenile mortality is typically enormous[41] and we initially expected higher juvenile abundance than recorded. In many pools, we were unable to collect juvenile fish despite high sampling effort covering all available microhabitats. However, we collected adults in the same pools later in the season and their age estimates suggested that juvenile fish must have been present in the pools during our previous sampling events. Higher catchability of adult fish, perhaps partially explained by a decrease of pool surface area, is a possible reason for this discrepancy. We suggest that juvenile killifish mortality is relatively low compared to the typical mortality rate in juvenile teleost fishes[41].

Male-biased mortality was clearly demonstrated in *N. furzeri*. Sex-ratio bias at the time of sexual maturation (20–40 days old) was small, but the proportion of males declined with age. At the age of 3 months, females always composed at least two thirds of the population and the sex ratio became even more biased in many populations (Fig. 4a). This is in agreement with results of Reichard *et al.*[18], who reported consistently female-biased adult *N. furzeri* populations and a seasonal increase in female bias between early and late adult stages[18]. *Nothobranchius orthonotus* populations also tended to be female-biased, though only one population (out of 4) demonstrated a decline in male numbers over their lifespan (Fig. 4b). However, analysis in the other three populations was limited to a maximum age of 2 months. Overall population size declined sharply after that age, leaving us unable to draw firm conclusions concerning seasonal trends in the sex ratio of *N. orthonotus* populations.

Both populations of *N. pienaari* unexpectedly exhibited an increase in the proportion of males, oscillating close to an equal sex ratio in one population but with a persistent female bias in the second (Fig. 4c). The increase in the proportion of males in *N. pienaari* populations was unexpected[18]. *Nothobranchius pienaari* prefer the grassy microhabitat at pool margins and pilot sampling at site LS2 (strong initial female bias) showed that *N. pienaari* males were more likely than females to be found in the marginal vegetation. More specifically, over 50 dipnet sweeps in each microhabitat we found the sex ratio to be male-biased (14:9; males:females) in the vegetated littoral zone compared to a clear female bias (1:10) in the open water. Consequently, the apparent increase in female relative abundance may arise from different sampling efficiency in different habitats[42] - females may have been more easily collected than males in the early stages of pool existence when dense grassy vegetation lined the pool margins. As gradual desiccation caused the water to recede from the vegetated pool margins, it is possible that males moved into the open water and were easier to capture.

Sex difference in lifespan and ageing is one of the unresolved phenomena of evolutionary biology and biogerontology. Males have a consistently shorter lifespan in many mammal species[43–45] while females are the shorter living sex in most birds[46]. A female-biased sex ratio is common in populations of small fish where sexual selection plays a prominent role in male reproductive success, such as in Neotropical annual killifish[47], Trinidadian guppies[42], European bitterling[48], as well as in other polygynous animals[46]. Whether this sex-bias in mortality is driven by selective predation on more conspicuous males[18,49] or reflects intrinsic male frailty[50] is currently not clear. While the presence of sex chromosomes[51] is a plausible explanation for the contrast between mammals and birds, fish have diverse systems of sex determination and are a promising system for understanding the roles of sexual dimorphism in size, colouration and behaviour, and hormonal regulation of metabolism in sex-specific lifespan and ageing[52].

At the interspecific level, we predicted that the proportion of *N. furzeri* in *Nothobranchius* spp. community would decrease over the season. Under benign laboratory conditions, *N. furzeri* is the shortest-lived species compared to its congeners[14,17]. While there was a trend towards selective disappearance of *N. furzeri* in some communities (Fig. 5: Ch2, LS2), the overall pattern was not statistically significant. We hypothesize that the natural lifespan of *N. furzeri* is relatively short compared to that observed in the laboratory and stronger changes in the species ratio would be observed in pools that persist longer than 2 months. Overall, *N. furzeri* is endemic to a relatively small region of the Limpopo and Chefu basins[32,53], where conditions are more arid than in other parts of the *Nothobranchius* spp. range[8,17]. In this region, *N. furzeri* is the most common annual species, often coexisting with the geographically more widespread *N. orthonotus* and *N. pienaari*[53]. Reichard *et al.*[53] suggested that the community pattern in species presence/absence is driven by abiotic factors rather than biotic interactions between *Nothobranchius* species and it is possible that *N. furzeri* is locally adapted to stronger fluctuation in habitat conditions compared to its sympatric congeners.

In conclusion, we demonstrated that natural *Nothobranchius* spp. populations declined strongly in abundance and were subjected to considerable mortality throughout the period of pool existence. The duration of aquatic phase varied across pools, with some populations experiencing only one month of post-hatching lifespan while others survived more than 4 months. Selective disappearance was apparent, with an increase in the sex-ratio bias in *N. furzeri* populations. Using several demographic measures, we demonstrated that the scope for selective mortality in *Nothobranchius* spp. populations before the pools desiccate can be substantial. Future research should address the functional aspects of condition-dependent mortality in wild populations of *Nothobranchius* species.

Methods

Study taxon. *Nothobranchius furzeri* is distributed throughout the semi-arid part of southern Mozambique and the adjacent part of south-eastern Zimbabwe[53]. Genetic analysis of wild populations revealed a relatively strong population genetic structure in *N. furzeri*, consisting of three major phylogeographic clades[31]. Populations from individual pools, often not more than a few kilometres apart, are significantly isolated[31,32]. Over much of its range, *N. furzeri* coexists with two congeneric species, *N. orthonotus* and *N. pienaari*[53,54] that represent important comparative taxa[10,55]. *Nothobranchius orthonotus* and *N. pienaari* have a wider geographical distribution that extends to central Mozambique[53]. The three species exhibit subtle differences in their habitat preference[54] and resource use[56,57].

Study sites. We performed longitudinal monitoring of 13 sites with *Nothobranchius* spp. between 17 January and 26 May 2016 (summarized in Table 1). The sites were distributed across the gradient in aridity (Supplementary Fig. 2) and rainfall predictability. More details on regional habitat conditions are provided elsewhere[18,53,54]. The sites were coded according to their affiliation to the *N. furzeri* geographic clade (Ch: Chefu region; LN: Limpopo North bank; LS: Limpopo South bank)[31]. Each site was visited between 3 and 14 times. We recorded pool area using a portable GPS device (Garmin GPSMAP® 64 s) by walking around the pool perimeter. The maximum depth of the pool was measured to the nearest 10 cm using a ruler. The presence of lungfish (*Protopterus* sp.), large predatory water bugs (Belostomatidae) and piscivorous birds was recorded (Supplementary Table 3) but their occurrence had no consistent geographic pattern.

Age. The age of the fish was estimated from otolith samples collected at the monitored sites (Supplementary Table 1). Three individuals (mixed sex) per species per site were collected and stored in 96% ethanol. Otoliths were dissected, polished and the number of daily increments was read by a commercial facility (Barcelona Otolith Reading Services, Spain). The estimated hatching date was calculated by subtracting the age estimate from the date of collection. The earliest estimate is given where different ages were recorded to provide a population level commencement of the hatching period. Otolith samples were not available for three sites (Ch3, LS3 and LS4) so we estimated their hatching dates on the basis of those of adjacent sites where otoliths were read successfully (for Ch3 from Ch1 and Ch2, for LS3 and LS4 from LS2, LS5 and LS6; Table 1).

Population density. We estimated Catch-per-Unit-Effort (CPUE) to compare fish density within pools between different sampling visits[58]. The CPUE typically consisted of 5 standardized hauls (5 m long) with a seine net (2.7 m long, 0.7 m deep, 4-mm mesh size), covering all habitats in the pool. Fewer hauls were completed when the pool was too small to avoid repeated sampling of the same area. The temporal dynamic in relative fish density was analysed for sites with at least three different CPUE sampling visits, providing data for 7 pools (Fig. 3).

Population size estimate. We employed two separate methods (Capture-Mark-Recapture and Catch-per-Unit-Area) to estimate the size of *Nothobranchius* spp. populations at 9 sites (Table 2). The Petersen method of population size estimate by Capture-Mark-Recapture (CMR) was used[58]. We marked fish by clipping a small part of their caudal fin (2 sites) or by injecting visible implant elastomer tags (Northwestern Marine Technology, USA) (5 sites). The upper lobe of caudal fin was clipped during the first CMR estimate, while the lower lobe of the caudal fin was clipped during the second CMR estimate, conducted 4 weeks later. Fins of *Nothobranchius* spp. regenerate quickly[59] and clipping from the first CMR estimate was almost undetectable after one month. Fish marked with elastomers were briefly anesthetized in a solution of clove oil and the tags were injected subcutaneously in two positions on their upper flank. Two marks were used to enable individual recognition for a separate study. After marking, fish were placed in fresh aerated water to recover and released back in the place of their capture. During marking and recovery, mortality was negligible (<2%). Fish marking was not size- or sex-selective; we marked all fish captured. The second sampling was conducted 15–24 h and 23–96 h after the first collection in populations marked using finclipping and elastomers, respectively. The entire pool area was always sampled, with the aim of collecting as many fish as possible over a 1–2 hour period.

The study pools were always isolated from other water bodies and migration was not possible. The Petersen formula for CMR population estimation: $N = (C \times M)/R$ was used, where N is the estimated population size, M is the number of fish captured and marked on the first sampling occasion, C is the number of fish captured on the second sampling occasion, and R is the number of individuals from the second sampling that had the mark. The 95% confidence intervals (CI) were calculated as $N \pm 1.96\times$ standard error (SE), where $SE = \mathrm{sqrt}(M^2 \times C \times (C - R)/R^3)$[60].

Catch-per-Unit-Area (CPUA) is a method that extrapolates the catch from a part of the sampling area (typically an enclosure) to the entire sampling area. We used CPUA at two relatively small and shallow sites containing only *N. furzeri* (LS3, LS4; Table 2). Three square enclosures (2.5 × 2.5 m (6.25 m^2) or 3 × 3 m (9 m^2) for LS3 and LS4, respectively) were formed using a dense mesh (1-mm mesh size) supported by metal rods driven to the substrate at each corner of the square. Once the enclosure was set, fish were collected using short dipnet sweeps (triangular dipnet 45 × 45 × 50 cm, 2-mm mesh size) over a 5–15 min period, depending on fish abundance. These sampling periods were repeated until the number of fish captured reached approximately 10% of the fish abundance during the first sampling period.

We estimated the initial population size in the enclosure by calculating the depletion regression of sampling period catch on cumulative catch (Leslie method): $C = q \times N_0 - q \times \sum C$, where C is the number of fish collected during each sampling session, q is catchability, N_0 is the original population size and $\sum C$ is the accumulated catch. The regression intercept equals $q \times N_0$ and the slope equals q[60]. The estimate from each enclosure (N_0) was independently extrapolated to the entire pool area, providing repeated estimates of the total number of fish in the pool (range given in Table 2).

Sex and species ratio. We employed additional sampling effort to the standard CPUE (pooled together as 'Total catch') to obtain a larger sample for estimates of population sex ratio and relative proportion of species present in the pool. We compared seasonal dynamics in sex ratio at 10 sites (17 populations of 3 species). Two rapidly desiccated sites (LS3, LS4) and one site with very low fish abundance (LS6) were excluded from the analysis. We also omitted sampling dates when juveniles were present, because small immature males cannot be reliably separated from adult females without dissection, providing potentially female-biased sex ratio estimates.

For the species ratio analysis, we selected sites that contained at least two species and where we collected at least 10 individuals per species on at least one sampling visit. This ensured that estimates were robust to random variation due to small sample size and enabled us to analyse *Nothobranchius* spp. communities from 7 sites, with 3–13 sampling events each (Fig. 5).

Legal approval. All work was carried out in accordance with relevant guidelines and regulations. Sample collection complied with legal regulations of Mozambique (collection licence: ADNAP-170/7.10/16) and research procedures were approved by the ethical committee of the Institute of Vertebrate Biology, in accordance with legal regulations of the Czech Republic. No experimental work was carried out; therefore, no experimental protocols to be approved were available.

Statistical analyses. All statistical procedures were conducted in the R environment (version 3.3.3, R Core Team). The response variables for respective analyses were Fish density, Sex ratio, and proportion of *N. furzeri* in the *Nothobranchius* spp. community. Explanatory variables were age (in days), site, species (for sex ratio analysis), level of sympatry (NF&NO, NF&NP, NF&NO&NP) (for proportion of *N. furzeri*). Data were not transformed. Defined models were visually inspected for any pattern in the distribution of residuals[61] and tested for significance of the focal fixed terms (at level $\alpha = 0.05$).

Fish density was the average number of *Nothobranchius* spp. individuals captured per haul in CPUE irrespective of species or sex. We fitted the data using generalised least squares models (GLS) with fish age, site identity and their interaction fitted as fixed factors. Different residual variance was allowed for each site.

Sex ratio was analysed as a bivariate variable - proportion of males caught per total catch (i.e. accounting for sample size) for a given species. We used binomial generalised linear mixed-effects models (GLMM) with logit-link function to test the effect of age on sex ratio for each species separately. We included both a random intercept (site) and slope (age) to account for repeated sampling. Similarly, the proportion of *N. furzeri* was a bivariate variable - the ratio of number of *N. furzeri* individuals to total fish caught at a site. A binomial GLMM with logit-link function was used to fit age and level of sympatry and both random intercept (site) and slope (age) were specified to correct for non-independence.

Data availability. Original data are available at Figshare repository (10.6084/m9.figshare.5863431), with no delay after submission of the files.

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Author Contributions

M.R. conceived and designed the study, M.V., J.Ž., M.P., R.B. and M.R. collected field data, M.V. analysed the data, and M.V. and M.R. drafted the manuscript. All authors revised the manuscript and approved its publication.

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PAPER 5: Reproductive senescence in the wild

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ORIGINAL PAPER

Limited scope for reproductive senescence in wild populations of a short-lived fish

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Abstract
Senescence in wild populations was long considered negligible but current evidence suggests that it is widespread in natural populations of mammals and birds, affecting the survival and reproductive output of older individuals. In contrast, little is known about reproductive senescence in species with asymptotic growth that can keep increasing their reproductive output as they grow older and larger. Using a cross-sectional study, we tested age-related decline in fecundity and relative allocation to reproduction in five wild populations of an annual killifish, *Nothobranchius furzeri* (Cyprinodontiformes). We did not detect any decline in absolute female egg production over their short lifespan in the wild. Relative fecundity (egg production controlled for female body mass) tended to decrease with age. This effect was driven primarily by a single population that survived 17 weeks, almost twice as long as the median persistence of the other four study populations. There was no decrease in relative ovary mass while in males, relative testes mass actually increased with age. Intra-population variation in relative ovary mass increased in older females suggesting heterogeneity in individual trajectories of female reproductive allocation. Overall, we demonstrate that annual killifish do not experience significant age-related decline in reproductive functions during their very short lifespan in the wild despite the marked deterioration of gonad tissue detected in captivity.

Keywords Reproductive ageing · Turquoise killifish · Gene by environment interaction · Plasticity · Population heterogeneity

Introduction

Senescence is a decline in fitness (survival and reproductive success) with chronological age (Shefferson et al. 2017). While it was long assumed that senescence cannot be observed in wild populations since individuals succumb to predation, diseases or harsh environmental conditions before senescence are manifested (Kirkwood and Austad 2000; Nussey et al. 2013), and recent evidence demonstrated that senescence in the wild is common (Clutton-Brock and Sheldon 2010; Nussey et al. 2013; Lemaître and Gaillard 2017). Senescence is modulated both by abiotic (Kawasaki et al. 2008; Caruso et al. 2017) and biotic conditions (Reznick et al. 2004). Hence, studying senescence in wild populations provides valuable insight into how different components of natural selection interact and mediate senescence—a challenging question for the evolutionary ecology of senescence that cannot be fully understood in captivity.

Reproductive senescence is age-related decline in reproductive functions or structures, such as the ability to allocate energy to reproduction, acquire a mate or provide sufficient parental care (Shefferson et al. 2017; Lemaître and Gaillard 2017). While these complex traits strongly determine individual fitness, data on reproductive senescence from wild populations have only recently become available (Shefferson et al. 2017). The pace and shape of reproductive senescence varies among taxa (Jones et al. 2014), with wild mammals and birds often experiencing decreases in reproductive functions later in

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life (Bérubé et al. 1999; Reed et al. 2008; Bouwhuis et al. 2009; Martin and Festa-Bianchet 2011). In contrast, reproductive senescence in fish and other animals with asymptotic growth is considered negligible or even negative (Vaupel et al. 2004), as their reproductive potential increases as they grow larger (Barneche et al. 2018). For instance, female garter snakes increase their reproductive effort with age while the probability of reproductive failure remains constant throughout their life (Sparkman et al. 2007) and large old female fish are considered the key individuals supporting population-level recruitment (Barneche et al. 2018). However, at least some fish species do experience reproductive decline (Reznick et al. 2006), including wild populations (Benoît et al. 2018), suggesting that reproductive senescence is relevant even for animals with asymptotic growth.

Individual variation may obscure the recognition of senescence at population level (Bouwhuis et al. 2009; Hämäläinen et al. 2014). Both improvement (given continuous growth) and decline (senescence) in reproductive functions are plausible with increasing chronological age (Hamel et al. 2018). Temporal dynamics in intra-population variation of the trait may therefore indicate differential survival among individuals of different condition (manifested by, for example, body mass, immune response or functional performance), effectively masking the incidence of functional and reproductive decline at the individual level (Hämäläinen et al. 2014). In addition, facing an imminent risk that threatens long-term survival may result in a late-life increase in reproductive effort, so-called 'terminal investment' (Bonneaud et al. 2004), whose manifestation is contingent upon individual condition and thus varies among individuals.

Nothobranchius furzeri is an iteroparous short-lived fish with very rapid life history adapted to ephemeral pools in the African savannah. The fish hatch synchronously when the pools are filled by seasonal rains (Reichard et al. 2017), grow fast, mature rapidly and then reproduce daily. All fish die when the pool desiccates (usually after 1–7 months), but populations may also disappear from the pool before its desiccation (Vrtílek et al. 2018a). During the dry phase, populations survive as resting embryos in dry pool sediment. In captivity, *N. furzeri* populations have a median lifespan of 4–7 months (Terzibasi Tozzini et al. 2013; Blažek et al. 2017) and exhibit senescence, including pathologies in reproductive organs (di Cicco et al. 2011) and decline in reproductive function (Kim et al. 2016). However, it is unclear whether reproductive senescence affects wild *N. furzeri*.

The abundance and density in wild *N. furzeri* populations decline with age (Vrtílek et al. 2018a) and mortality is stronger in males (Reichard et al. 2014; Vrtílek et al. 2018a). Importantly, the growth and maturation of *N. furzeri* are much faster in wild compared to captive conditions, with the onset of reproduction at the age of 3–5 weeks in captivity (Blažek et al. 2013, 2017; Cellerino et al. 2016) but as early as 2 weeks in wild populations (Vrtílek et al. 2018b). This is adaptive as the pools often desiccate rapidly (1–4 months after being filled) (Vrtílek et al. 2018a, b). Hence, natural selection should favour investment into early reproduction (Sibly and Calow 1986; Kirkwood and Rose 1991), despite its costs in terms of later reproduction or survival (Boonekamp et al. 2014; Lemaître et al. 2015; Zhang and Hood 2016), apparently manifested in rapid maturation and short intrinsic lifespan in *N. furzeri* (Valdesalici and Cellerino 2003; Blažek et al. 2013; Vrtílek et al. 2018b). Consequently, we predicted earlier onset and greater magnitude of reproductive senescence in wild populations of *N. furzeri* compared to captive conditions where no declines in gonad mass, clutch size and fertilisation rate were observed in fish aged 20–23 weeks (Blažek et al. 2017). Increased lipid retention in the liver has been suggested as a potential marker for senescence in captive *N. furzeri* (di Cicco et al. 2011), but lipids are also utilised in vitellogenin production in the liver (Selman et al. 1993) and we explored their role in mediating fecundity and reproductive allocation.

In the current study, we tested the existence of reproductive senescence in replicated wild populations of *N. furzeri*. We predicted that fecundity (reproductive output) and relative allocation to reproductive tissue (gonad mass) would decrease with age. We further predicted that intra-population variation in reproductive allocation would increase with age due to individual variation in response to challenging conditions in the wild. In addition, we tested how liver lipid retention level is associated with sex, age and reproductive allocation.

Methods

Study populations

We followed five populations of *Nothobranchius furzeri* (distributed across the entire species range in southern Mozambique) over their natural life cycle throughout the rainy season (17 January 2016–26 May 2016) (Table 1). Samples were collected at the age of 17–121 days (2.5–17 weeks) (collection licence issued by Mozambican Ministry of Sea, Inland Waters and Fisheries: ADNAP-170/7.10/16). In four populations, two seasonal samples were collected, with the first sample at 17–40 days and the second sample 47–74 days (with an interval of 19–34 days between samplings). The four populations had natural lifespan of 72–110 days. The fifth population had a lifespan of > 121 days and provided four seasonal samples (at 40, 70, 108 and 121 days).

Fish were always collected between 07:00 and 08:00 AM (i.e. shortly after sunrise), before the start of reproductive activity (Haas 1976; Polačik et al. 2016). A sample of fish was captured using a seine net (2.7-m long, 0.7-m deep, 4-mm mesh size) and a target subsample was randomly selected, sacrificed by an overdose of clove oil, stored in Baker solution

Table 1 Overview of populations studied. 'Clade' is a phylogeographic lineage of *N. furzeri* (Bartáková et al. 2013), 'hatching' is the date estimated based on otolith readings, 'final age' gives the number of days between estimated hatching and pool desiccation. Population Ch1 survived beyond our fieldwork period and fish from population LS5 died between days 75–86, before the pool desiccated. '*N* samples' gives number of sampling dates and, in parentheses, the number of females and males collected in total. Note that only a subsample was used for liver lipid retention analysis (see "Methods")

Site	Coordinates	Clade	Hatching	Final age	*N* samples (F, M)
Ch1	E 32° 53′ 54″, S 22° 16′ 33″	Chefu	26. 1. 2016	> 121	4 (22, 16)
Ch2	E 32° 43′ 38″, S 22° 33′ 17″	Chefu	26. 1. 2016	72	2 (15, 10)
LS1	E 32° 22′ 45″, S 24° 11′ 40″	Limpopo South	18. 3. 2016	59	2 (17, 10)
LS2	E 32° 36′ 55″, S 24° 18′ 15″	Limpopo South	10. 1. 2016	110	2 (26, 10)
LS5	E 32° 46′ 30″, S 24° 24′ 59″	Limpopo South	11. 1. 2016	86	2 (24, 10)

and returned to the laboratory for dissection. We aimed to collect 10 females and 5 males per sampling, though sample size varied due to fish availability (Table 1). Sampling frequency and size were kept to a minimum so as not to interfere with our long-term monitoring of the population dynamics of each study population. The study populations were monitored regularly, with 3–14 visits to record environmental parameters (temperature, conductivity, pool surface area, water depth, vegetation cover). The details of habitat conditions and population size dynamics are provided elsewhere (Vrtílek et al. 2018a). In general, pool area ranged from 780 m^2 (Ch2) to 3300 m^2 (Ch1) and declined seasonally. Population abundance and population density declined over the study period (Vrtílek et al. 2018a). Water temperature varied between 16.8 and 38.0 °C, with a pronounced diel periodicity that was much stronger than seasonal dynamics. All work was carried out in accordance with relevant guidelines and regulations.

Fish dissection and histology

In the laboratory (IVB, Brno), fish were dissected and their total body mass, eviscerated body mass and gonad mass were weighed to the nearest 0.001 g. The number of mature (ovulated) oocytes was counted as a measure of current female fecundity. *Nothobranchius furzeri* ovulate overnight and lay their eggs singly during the following day. Hence, the number of mature eggs in the morning represents the investment in reproduction for a particular day. Livers were extracted in a subset of sampled fish (57 out of 103 females and 54 of 55 males), weighed, stored in Baker solution and sent to the laboratory at FLI in Jena for histological sectioning to estimate the level of cellular lipid retention (steatosis). At the FLI, livers were embedded in Paraplast, sectioned (5 μm) and stained in H&E. Lipid retention was scored blind to sample identity using the scale of Di Cicco et al. (2011) (score 0–3, 0: no lipid retention in liver cells, 1: minor diffused and localised lipid retention with some cellular swelling present, 2: moderate diffused or localised lipid retention, 3: substantial lipid retention) (Fig. S1).

Fish age

For each population, the age of the fish was estimated from otolith samples. Three individuals (mixed sex) from each study population were collected prior to the first collection of samples for histology (age 8–26 days) and stored in 96% ethanol. Age estimates from younger fish are more precise (Reichard et al. 2017) and a single age cohort was present in each pool (Vrtílek et al. 2018a). Otoliths were dissected and polished and the number of daily increments was read by a commercial facility (Barcelona Otolith Reading Services, Spain). Date of hatching was calculated by subtracting the estimated age from the date of collection and subsequently used to calculate the age of fish in respective samples. This method was previously validated using captive individuals of known age (Polačik et al. 2011). The estimated age of fish from the same population was within 1 day, except for a single fish that was 3 days older than the rest of the sample (population LS2). The earliest estimate was used when different ages were recorded among individuals within a population to provide a population-level hatching date (Table 1). The full description of the age estimation method is provided in Reichard et al. 2017.

Statistical analysis

Change in absolute fecundity (number of mature oocytes) was tested using Generalized Linear Mixed Models (GLMM) with negative binomial error distribution to account for a major overdispersion in residuals in GLMM with a log-link, with age as a continuous fixed factor. The population ID was modelled as a random intercept, with age as a random slope. The same approach was used to analyse relative fecundity, with body mass added as a covariate. To test the change in allocation to reproductive tissue (gonad mass corrected for body mass), we fitted a General Least Squares model (GLS) with compound symmetry correlation structure defined by population ID. The GLS modelling approach is equivalent to Linear Mixed Models (LMM) with population treated as a

random intercept (Pekár and Brabec 2016), but additionally enabled correction for heteroscedasticity that was present in our dataset. The sexes were analysed separately, because ovary mass and testes mass are known to differ markedly. Age was a continuous fixed effect, with body mass used as a covariate in the analysis of relative allocation to reproductive tissue.

We calculated the coefficient of variation (CV, the ratio of the standard deviation in the sample to the mean value) in the male and female Gonadosomatic Index (relative allocation to reproductive tissue calculated as a proportion of gonad mass per eviscerated body mass) for each age by population combination (the first and second samples) and compared the level of intra-population variation between younger and older fish using a non-parametric Wilcoxon signed rank test. Given our specific directional prediction (individual heterogeneity in senescence can increase but not decrease intra-population variation), we used one-tailed version of the test.

We tested the association between the level of cellular lipid retention in the liver and the number of mature oocytes using GLMM with negative binomial error distribution with a log-link and the random intercept and slope. We tested the association between lipid retention in liver and ovary mass using GLS. Cellular lipid retention score (ordinal scale with four levels, 0 to 3) was added as a fixed variable. We also performed exploratory analysis of liver cellular lipid retention between the sexes with respect to age including their interaction using Poisson GLMM with log-link with population ID as a random intercept. All explanatory variables were zero-centred (mean = 0) and scaled to one unit of variance (1 standard deviation). Residuals were tested for overdispersion and negative binomial error distribution was employed when overdispersion was detected. We examined heteroscedasticity in all models and corrected for it where necessary (ovary mass GLS model). We removed a single female outlier (population Ch1, age 40 days) from all analyses because she had atrophied ovaries (ovary mass 0.006 g; interquartile range of ovary mass was 0.082–0.164 g and the second smallest ovary was 0.034 g). We used the packages 'nlme' v3.1-137 (Pinheiro et al. 2018), 'lme4' v1.1-18-1 (Bates et al. 2015) and 'MuMIn' v1.42.1 (Barton 2018) in R v3.5.0 (R Core Team 2018) for all statistical analyses. The data and R code used for the analysis are publicly available at Figshare repository (doi: https://doi.org/10.6084/m9.figshare.7321751).

Results

Fecundity

The number of mature oocytes did not decrease with female age (GLMM, age -0.054 ± 0.057 (estimate ± standard error), $z = -0.96$, $P = 0.338$; $N = 103$) (Fig. 1a). Relative fecundity (i.e. number of mature oocytes with body mass as a covariate) tended to decline with age, though the effect was not statistically significant (GLMM, age -0.145 ± 0.076, $z = -1.91$, $P = 0.056$; body mass 0.398 ± 0.070, $z = 5.72$, $P < 0.001$, $N = 103$).

Allocation to reproductive tissue

Relative ovary mass did not significantly decrease with female age (GLS, age -0.008 ± 0.005, $t = -1.56$, $P = 0.121$; body mass covariate 0.053 ± 0.006, $t = 8.33$, $P < 0.001$; $N = 103$) (Fig. 1b). Relative testes mass increased, rather than decreased, with male age (GLS, age 0.002 ± 0.001, $t = 2.90$, $P = 0.005$; body mass covariate 0.006 ± 0.001, $t = 8.12$, $P < 0.001$; $N = 55$) (Fig. 1c).

The variation in relative reproductive allocation among females within a population (CV of Gonadosomatic Index per sample) was low in young females, but increased with age (left-tailed Wilcoxon signed rank test: $z = 2.02$, $P = 0.031$; $N = 10$) (Fig. 2a). In contrast, the variation in Gonadosomatic Index among males did not increase in older males (left-tailed Wilcoxon signed rank test: $z = 1.48$, $P = 0.938$; $N = 10$) (Fig. 2b).

Lipid retention in livers

The number of mature oocytes tended to be negatively associated with the level of liver lipid retention (GLMM, lipid retention score -0.100 ± 0.056, $z = -1.79$, $P = 0.073$; $N = 57$). Relative allocation to reproductive tissue (with age and body mass as covariates) was not associated with lipid retention in females (GLS, lipid retention score -0.005 ± 0.004, $t = 1.34$, $P = 0.187$; $N = 57$) or males (0.001 ± 0.001, $t = 1.53$, $P = 0.132$; $N = 54$). In general, lipid retention in livers was higher in males than in females (GLMM 1.135 ± 0.186, $z = 6.09$, $P < 0.001$; $N = 111$), but did not change with age (0.104 ± 0.102, $z = 0.98$, $P = 0.329$) and there was no interaction between sex and age ($z = -1.13$, $P = 0.259$ in the full model).

Discussion

Senescence was long assumed to be manifested only in captive conditions where the elimination of external sources of mortality enables animals to live long enough to experience senescent decline in functional traits (Kirkwood and Austad 2000). Recent research on natural, free-ranging populations of birds and mammals has confirmed that particular traits, including reproductive traits, are commonly subject to senescent declines in the wild (Nussey et al. 2013; Mourocq et al. 2016). For example, long-term demographic studies have demonstrated decreased lamb production in older wild bighorn ewes (Bérubé et al. 1999) and lower numbers of fledglings in older

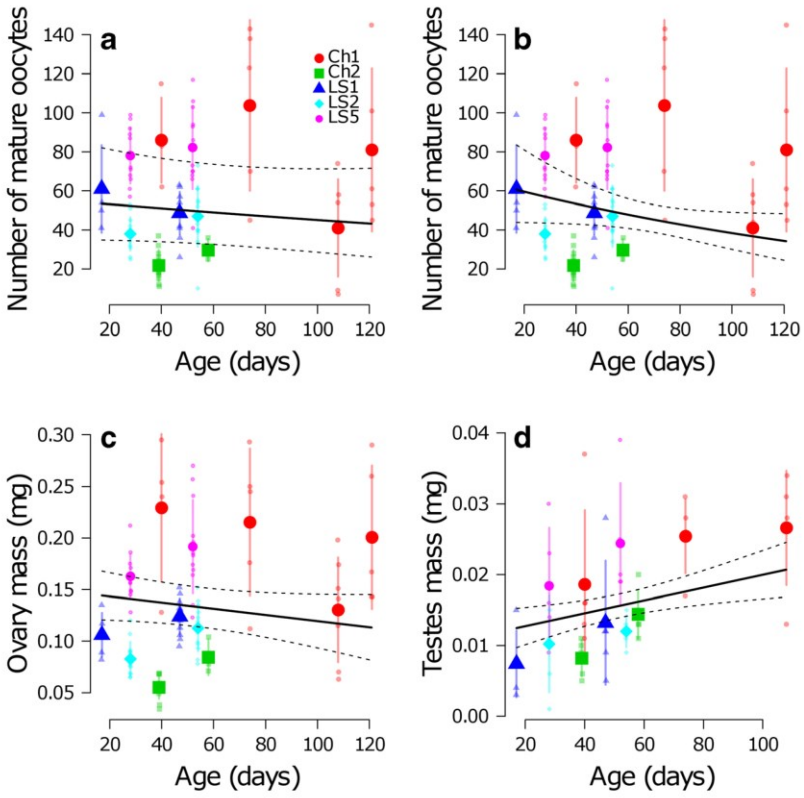


Fig. 1 Age-related reproductive output in 5 wild populations of *N. furzeri*—**a** absolute female fecundity (number of mature oocytes uncorrected for body size), **b** relative female fecundity (predicted effect of age on number of mature oocytes when accounting for body mass as a covariate), **c** female and **d** male relative gonad mass (with body mass as a covariate). The horizontal solid line shows the predicted relationship between each response variable and age, with dashed lines indicating 95% confidence intervals. The small points in the background are raw values, the solid points represent sample-specific mean values and the vertical error bars denote one standard deviation

great tits (Bouwhuis et al. 2009). A few recent studies on fish and other animals with asymptotic growth have also reported marked senescence in reproductive functions in semi-natural (Massot et al. 2011) and natural populations (Benoît et al. 2018), suggesting that reproductive senescence may be more widespread in nature than is generally recognised. Here, we focus on reproductive senescence in wild populations of *N. furzeri*, a species with an extremely condensed life history. Our cross-sectional study demonstrated no significant age-related decline in fecundity or relative gonad mass among *N. furzeri* females. Intra-population variation in relative gonad mass was much higher in older females but this variation was not associated with liver lipid retention. In males, relative gonad mass even increased with age.

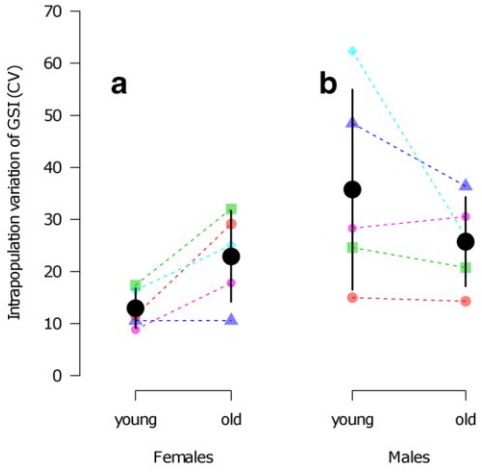


Fig. 2 Intra-population variation in Gonadosomatic Index (GSI) in young and old females (**a**) and males (**b**). The coefficient of variation (CV) was calculated as a measure of variability within each sample and is shown by population-specific colour and symbol, corresponding to the specifications used in Fig. 1. The large black points illustrate mean values across the populations. The error bars represent one standard deviation

At the physiological level, there is evidence of reproductive senescence from captive populations of female *N. furzeri* and closely related species, but at an age far beyond 10 weeks that is the average population-level survival in the wild (Vrtílek et al. 2018a). In two laboratory strains of *N. furzeri*, follicular degeneration and fibrosis was detected in approximately 30% of old females (aged 16 and 28–32 weeks for the respective strains), while the incidence of neoplasia in ovaries was relatively low (< 10%) compared to other organs (di Cicco et al. 2011). Old (12 months) *Nothobranchius guentheri* females from a laboratory population suffered higher incidence of atretic oocytes and lower production of vitellogenins (Liu et al. 2017), glycolipoproteins that supply ovulating eggs with yolk (Selman et al. 1993; Reading and Sullivan 2011). This indicates a decline in the ability to efficiently transform the available resources into mature eggs, potentially explaining our observation that older females did not decrease their

relative reproductive allocation (relative gonad mass) while their relative fecundity (number of mature eggs corrected for body size) tended to decline. This tendency in reproductive fecundity decline is unlikely due to decreased resource availability for ovulating older females, as condition factor (Fulton Index) in females did not decrease with age (Fig. S2). In addition, a previous laboratory study demonstrated that food-deprived female *N. furzeri* reduced reproductive allocation along with fecundity (Vrtílek and Reichard 2015). It is possible that a particular (yet unidentified) pathway of female reproductive physiology becomes altered at older age, resulting in reduced production of mature eggs.

There was no evidence of a decline in reproductive allocation and fecundity with age in captive *N. furzeri* populations (Blažek et al. 2017) estimated at the age of 10 and 20 weeks. In the longer-lived *N. orthonotus* and *N. pienaari*, estimates over a period of 50–60 weeks demonstrated clear age-related declines in fecundity (Blažek et al. 2017). The two species are sympatric and closely related to *N. furzeri* but their lifespan in captivity is approximately 50–75% longer (Terzibasi Tozzini et al. 2013; Blažek et al. 2017). Reproductive allocation did not decline in any of the eight populations of four *Nothobranchius* species tested by Blažek et al. (2017), in agreement with the outcome of the present study.

In wild populations, we detected a tendency for relative fecundity to decline with age. This pattern was primarily driven by a single population that was sampled over a period of 17 weeks, while the trend was inconclusive in the remaining four populations monitored for a considerably shorter time (6–11 weeks). Twenty-week-old females in captivity did not exhibit senescence in egg production (Blažek et al. 2017). We hypothesised that rapid reproductive senescence in wild populations of *N. furzeri* might arise from a greater trade-off between early development and late-life fitness. Under natural conditions, juvenile development is extremely rapid, with fish commonly reaching maturity in 2 weeks after hatching (Vrtílek et al. 2018b) while in captivity, the juvenile period can be twice as long (3–5 weeks) (Blažek et al. 2013, 2017; Cellerino et al. 2016). The costs of rapid growth and high investment into early reproduction are paid later in life, either in terms of decreased survival (Descamps et al. 2006; Boonekamp et al. 2014) or reduced late-life reproduction (Nussey et al. 2006; Reed et al. 2008). Our analysis does not support the hypothesis that a decline in egg production is manifested in the wild, at least over ecologically relevant longevity.

Intra-population variation in female relative reproductive allocation increased with age in the study populations, suggesting considerable individual heterogeneity. Given that wild *N. furzeri* females maintained the same reproductive allocation on average, increased late-life intra-population variation in relative reproductive allocation suggests that reproductive fitness improved with age in some females but declined in others. The level of liver lipid retention could be an appropriate marker of condition and positively correlate with female fecundity. While a high level of lipid retention in the livers is pathological in mammals (including humans: Vernon et al. 2011), the liver serves as a lipid storage organ in many fishes (Sheridan 1988). We observed sex differences in lipid retention in livers in wild fish, a feature that has been repeatedly observed in captive *N. furzeri* (di Cicco et al. 2011; Cellerino et al. 2016). We speculate that this represents a sex-specific pattern of lipid metabolism. Female fish utilise lipids stored in livers by allocating them to vitellogenins, representing the major resource for developing embryos (Selman et al. 1993; Reading and Sullivan 2011). We found that the most fecund females tended to have relatively less lipid in their liver, but there was no relationship between lipid retention in livers and female relative gonad mass. This might suggest that females may utilise stored lipids for egg production (increased resource use). We fully acknowledge that we made no particular a priori prediction of the direction of the relationship and this correlation should be taken with caution. The increased intra-population variation in female reproductive allocation could also have been a consequence of low male density, with some females accumulating eggs that were not spawned on the previous day (Fig. S3). Overall, the elevated inter-individual variation in reproductive traits at older age demonstrates the importance of studying intra-population variation rather than just the population mean (Hayward et al. 2015; Hamel et al. 2018) and points towards individual-specific patterns of reproductive senescence in female *N. furzeri* that shall be further explored in longitudinal studies.

The sexes differ in the magnitude and trajectories of senescent declines, including reproductive traits (Cornwallis et al. 2014). In some species, males are able to maintain high reproductive success over their lifetime (Hayward et al. 2015; Lemaître and Gaillard 2017), but males of other species exhibit steep reproductive senescence (Dean et al. 2010). We focused on a single measure of male reproductive ability—the relative mass of testes. While large testes result in high male reproductive success in species with promiscuous mating systems (Preston et al. 2003), the cost of sperm production is higher than usually assumed (Wedell et al. 2002; Smith et al. 2009), making senescent decline in testes size plausible (Hayward et al. 2015; Johnson et al. 2018). In our study, wild male *N. furzeri* showed a marked increase in relative testes mass with age. Captive-bred male *N. furzeri* maintained relatively high fertilisation success at the age of 20 weeks (Blažek et al. 2017), suggesting they did not suffer from reproductive senescence. Also, at the histological level, only rare and limited testes pathology was observed at the age of 11 weeks (di Cicco et al. 2011). Testis fibrosis, degeneration and atrophy in various combinations and degrees affected most male *N. furzeri* only at the age of 28–32 weeks (di Cicco et al. 2011), which is beyond the maximum lifespan of males in wild populations.

Overall, we demonstrated that wild females of extremely short-lived fish species do not suffer significant age-related decline in fecundity during their short natural lifespan of 4 months, despite a tendency for reduced relative fecundity over that period. Individual variation in reproductive allocation among females within each population increased with age, but this heterogeneity was not associated with the extent of lipid retention in livers. Survival in natural populations of *N. furzeri* appears too short for reproductive senescence to be manifested despite its expression in captivity.

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Author contributions MR conceived and designed the study, MV, JŽ, MP, RB and MR collected field data, AC conducted the histological analysis of livers, MV analysed the data, and MV and MR drafted the manuscript. All authors revised the manuscript and approved its publication.

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Reproductive senescence in a short-lived fish

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Abstract

1. Reproductive senescence is an age-associated decline in reproductive performance, which often arises as a trade-off between current and future reproduction. Given that mortality is inevitable, increased allocation into current reproduction is favoured despite costs paid later in life.
2. This assumption is violated in organisms with post-maturity growth whose reproductive output increases long after maturity. While reproductive senescence is frequently studied in animals with determinate growth at maturity, such as insects or mammals, we have very limited understanding of reproductive senescence in organisms with an extensive post-maturity growth period.
3. The fact that many post-maturity growers experience strong adult mortality leads to conflicting expectations for reproductive senescence. The aim of this study was to investigate how co-occurrence of rapid life history and post-maturity growth mould reproductive senescence in a short-lived killifish, *Nothobranchius furzeri*, using longitudinal data on laboratory and wild-type populations.
4. We followed the individual fecundity, fertility and fertilization of 132 singly housed fish from the perspectives of chronological and biological age. At the onset of senescence, the sex-specific contribution to decrease in fertilization capacity was investigated. Allocation trade-offs were estimated through the association between reproductive parameters and life span, and between early-life and late-life fecundity.
5. We demonstrate that female fecundity increased steadily after maturity and reproductive senescence occurred long after the growth asymptote. The prime age for fecundity coincided with 50% female survival and consequent decline in fecundity implies an association with somatic deterioration. Reproductive senescence in fertilization rate was stronger in females than in males. Females with high early fecundity experienced a long life span and high late-life fecundity, discounting the role of allocation trade-offs in reproductive senescence.
6. The present study reports a clear case of reproductive senescence in a fish with a long post-maturation growth period, unusually rapid development and short life span. The onset of reproductive senescence was postponed compared to animals that cease growing at sexual maturity. Fish and other animals with post-maturity growth have long been considered insusceptible to ageing but this conclusion may be related to the previous lack of longitudinal data rather than to the absence of reproductive senescence in such organisms.

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KEYWORDS
annual killifish, indeterminate growth, life-history evolution, *Nothobranchius furzeri*, reproductive ageing, senescence

1 | INTRODUCTION

Reproductive senescence is an age-related decline in reproductive performance. This decline is predicted by evolutionary theories of ageing (Lemaître & Gaillard, 2017), either as an inevitable by-product of early investment into reproduction (Antagonistic Pleiotropy theory: Williams, 1957; Disposable Soma theory: Kirkwood, 1977) or persistence of late-acting detrimental mutations due to the weakened strength of natural selection after maturity (Medawar, 1952; Williams, 1957). Nevertheless, the predicted decrease in the strength of natural selection after maturity may not be universal (Finch, 1998; Vaupel et al., 2004).

In organisms with continuous post-maturity growth, the strength of natural selection is expected to persist long after maturity, since their fitness is closely linked to their body size (Finch, 1990; Vaupel et al., 2004). Consequently, these organisms are predicted to have evolved a delayed onset of senescence long after maturity (Medawar, 1952; Vaupel et al., 2004). Post-maturity growth is typical in molluscs, fish and reptiles (Barneche et al., 2018; Charnov et al., 2001; Heino & Kaitala, 1999) and, unlike birds, mammals or insects, their asymptotic size is not reached at the age of maturity but is postponed until long after sexual maturation (Blažek et al., 2013; Charnov et al., 2001; Heino & Kaitala, 1999). Some organisms with post-maturity growth are short lived (Eckhardt et al., 2017; Reichard & Polačik, 2019) and express rapid life history which predicts an accelerated rate of senescence (Jones et al., 2008). Hence, these organisms provide interesting models to examine the interplay between contrasting adaptations arising from post-maturity growth (postponed senescence) and fast life (rapid senescence).

A captive environment provides protection from multiple environmental factors (Mason, 2010). Hence, animals in captivity often live longer and due to the absence of environmentally driven mortality, they can reach the senescent stage even when this is not commonly observed in the wild (Kirkwood & Austad, 2000; Tidière et al., 2016). By contrast, keeping populations for multiple generations under relaxed natural selection in a captive environment can affect organismal life histories including reproduction and senescence rate (Heath et al., 2003; Sgro & Partridge, 2000). Despite detrimental effects of captivity on reproduction, captive populations may outperform wild populations in reproductive parameters due to selection for high reproductive performance (Miller et al., 2002). There are contrasting assumptions from ageing theory of how captivity might shape organismal senescence. Williams (1957) suggested that a reduction in the environmentally driven mortality would lead to delayed maturity and life span extension while under Positive pleiotropy, the reduced purifying effect of condition-dependent mortality in captivity would contribute to low overall fitness in a captive-adapted population (Kimber & Chippindale, 2013; Maklakov et al., 2015). In addition, high allocation to reproduction may come with costs to survival, leading to a shorter life span of a more fecund population (Kirkwood, 1977). Thus captivity-adapted and wild populations differ in their history of senescence-shaping environmental mortality and comparison of senescence rate between such populations under common garden conditions may provide important insights into the evolution of senescence (Reznick et al., 2004; Sgro & Partridge, 2000).

Environmental mortality is a strong determinant of life span in small fish species (Blažek et al., 2017; Reznick et al., 2004), but little is known about reproductive senescence in fish. It is widely assumed that fish have evolved delayed senescence (Hamilton, 1966) or lack senescent deterioration at all (Finch, 1990; Vaupel et al., 2004). Given that fish experience a long period of post-maturity growth but often live at a fast pace (Winemiller & Rose, 1992), studies on ageing in fish can deliver new insights into the evolutionary mechanisms of reproductive senescence (Reznick et al., 2002, 2004). *Nothobranchius furzeri* are small fish (<7 cm) that express adaptions to a life cycle enabling them to occupy seasonally desiccating savanna pools in south-east Africa (Reichard & Polačik, 2019). Given their naturally short life span, they have become a vertebrate model for studies on ageing (Cellerino et al., 2016; Hu & Brunet, 2018). Constraints on their life span are predicted to produce strong selection for high allocation into early reproduction, irrespective of its potential future costs (Kirkwood, 1977). In conflict with this prediction, no functional reproductive senescence has been detected in captive (Blažek et al., 2017) or wild *N. furzeri* populations (Vrtílek, Žák, Blažek, et al., 2018), in line with expectations for organisms with a long period of post-maturity growth (i.e. *N. furzeri* mature at as little as 55% of their asymptotic body size, Blažek et al., 2013). Previous studies have used a cross-sectional design and, hence, are prone to be affected by selective disappearance (Nussey et al., 2008). Thus ideally a longitudinal study is required to test whether short-lived species, such as *N. furzeri*, are subject to functional reproductive senescence.

In the present study, we characterize reproductive senescence in two populations of *N. furzeri*, using detailed longitudinal data based on regular measurements from the same individuals. We predicted that despite post-maturity growth, the fast pace of life of *N. furzeri* and the protected environment of a laboratory setting would allow expression of reproductive senescence.

2 | MATERIALS AND METHODS

2.1 | Experimental fish and conditions

We used a common garden experiment with two populations of *N. furzeri*, laboratory (GRZ) and wild-type (WT), to compare their

demographic parameters in relation to reproductive senescence. The GRZ population has been held in captivity for >100 generations, and despite a broadly similar genetic background to the WT population (Bartáková et al., 2013), it is highly inbred (Cellerino et al., 2016; Reichwald et al., 2015). The GRZ population originated from the driest part of the natural range of *N. furzeri* in Zimbabwe and was collected in 1968 (Jubb, 1971). The WT strain is of recent wild origin (collected in 2011 in southern Mozambique (GPS 21°52′24.84″S, 32°48′2.34″E, approximately 100 km from the site of collection of the GRZ strain), relatively outbred (Cellerino et al., 2016) and was maintained in our laboratory (Institute of Vertebrate Biology [IVB], Czech Academy of Sciences) under the code MZCS NF 222 for six to eight generations prior to the experiment (permit 70084/2016-MZE-17214).

All experimental fish hatched in September 2017 at the IVB following a standard protocol (Polačik et al., 2016). Hatched fish were housed in communal tanks until the age of 5 weeks, when the sex of all fish could be determined (Polačik et al., 2016). Thereafter, 132 fish (65 GRZ and 67 WT, approximately 1:1 sex ratio) were placed in individual 2-L tanks in three separate recirculation systems (Aquamedic) where they were kept until natural death. For the analyses of reproductive senescence, only females which produced at least one clutch of eggs were used (i.e. we have not included seven GRZ females that died prior to producing their first clutch). Fish from each strain were assigned to alternate tanks in predetermined positions with equal proportions of WT and GRZ fish across recirculation units. Fish were fed twice each day (9:30, 17:00) ad libitum (amount consumed within 5 min) with frozen bloodworm. Prior to each feeding, the tank floor was cleaned by siphoning and the health status of every fish was visually inspected. Water temperature was maintained at 27°C (range 25–29°C), water conductivity was 250–300 μs and light regime was 14:10 L:D.

2.2 | Measurement of reproductive traits

Killifish have external fertilization, enabling us to separate and non-invasively estimate the production of ovulated oocytes and the fertilization rate. In our study, we estimated age-specific fecundity (number of eggs laid; estimate of female reproductive allocation), fertility (number of fertilized eggs laid; estimate of parental reproductive fitness), fertilization rate (proportion of eggs successfully fertilized; combination of male and female gamete quality), sexual maturation (age at first oviposition), lifetime reproductive output (sum of all eggs laid) and maximum clutch size (number of eggs in the most fecund oviposition over an individual's lifetime). Every week, pairs were placed in 2-L plastic containers prior to morning feeding and allowed to spawn on a sand substrate for 2 hr, which is sufficient for females to release all ovulated eggs representing their daily fecundity (Polačik et al., 2016). Spawned eggs were left in the plastic container for 24 hr (to allow egg-shell hardening) and then sieved from the sand (Polačik et al., 2016). Fertilized and unfertilized eggs were counted under a dissecting microscope (10× magnification). Fertilized eggs were identified by the presence of a perivitelline space (Polačik et al., 2016). After oviposition, the pair was photographed in a shallow plastic dish with a reference scale. Body size (length without caudal fin) was measured from photographs using ImageJ v 1.48 (USA, https://imagej.nih.gov).

A second, non-experimental oviposition was realized every week (3 days after the experimental oviposition) throughout the post-maturity life span. This was to prevent ovary malfunction (i.e. loss of capability to release ovulated eggs) which may arise when females are not allowed to spawn daily (Polačik et al., 2014) as is typical for *N. furzeri* in the wild (Žák et al., 2019). Despite this precaution, 6 WT (18%) female deaths were suspected to be the result of ovary malfunction, a substantial reduction from 82% observed in an earlier study during which females were not allowed to spawn over their life span (Polačik et al., 2014). Spawning pairs were stable, but in the case of death, a missing partner was replaced with a fish that had similarly lost its mating partner.

2.3 | Experimental pairing of young and old fish

At the age of 31 weeks, when fertilization rate was low (Figure 1d), 10 pairs of WT fish were combined with 10 pairs of young (8 weeks old) fish from the same population to determine the sex- and age-specific declines in reproductive function. Young fish originated from social tanks (10 males and 15 females in a 60-L tank). They were allowed to mate freely in their social home tanks, but isolated in two single-sex aquaria 4 days prior to first experimental spawning to standardize their reproductive state. Every fish (20 old and 20 young) spawned twice in the experiment, once with an old and once with a young partner, in a random order. This design resulted in four treatment combinations (old female–old male, old female–young male, young female–old male and young female–young male). The second spawning followed 3 days after the first. After the first oviposition, young fish were placed in single-sex trios (small, medium and large fish) in separate 35-L tanks to permit individual identification. Only the fertilization rate was compared between old and young fish, as fecundity of females from social tanks is typically higher than in individually housed fish, irrespective of age (J. Žák, pers. obs.).

2.4 | Life span and survival analysis

All statistical procedures were performed in the R environment v 3.6.1. (R Core Team, 2019).

Group-specific survival was visualized with Kaplan–Meier plots and analysed using a nonparametric Log-rank test from the SURVIVAL v 2.44.1.1 package (Therneau & Grambsch, 2000). To determine the best predictor of female life span, we included early-life reproductive effort (cumulative fecundity from the first three clutches), maximum fertilization rate (maximum value of fertilization rate for each individual

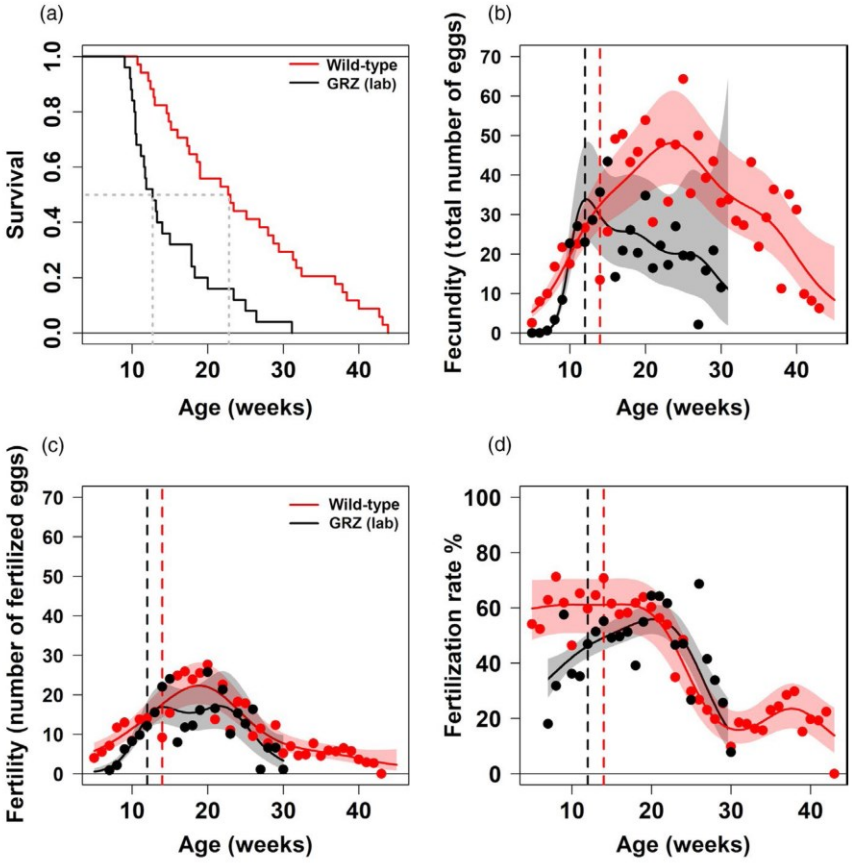


FIGURE 1 Survival and dynamics of reproductive parameters with chronological age in two populations of *Nothobranchius furzeri*. Age-specific female survival (Kaplan–Meier; a), fecundity (b), fertility (c) and fertilization rate (d) of the wild-type (WT; red) and laboratory GRZ (black) populations of the *N. furzeri*. Fecundity, fertility and fertilization rate curves are modelled with a GAM. Shading represents 95% confidence intervals. Points are produced by GLMM with age as a fixed factor and ID-female as a random factor to account for repeated measurements. Grey-pointed lines in (a) represent median survival. Black (GRZ) and red (WT) vertical-dashed lines in (b–d) are age when female-specific growth rate was not statistically different from 0 (see Figure S2 in Supporting Information). Male age-specific survival is presented in Figure S3

to provide an estimate of egg quality produced by each female), juvenile growth (size at 8 weeks) and population ID as explanatory variables (all continuous variables were zero-mean centred with standard deviation of 1, Schielzeth, 2010), and modelled as a Gamma GLM. We originally also included age at maturity as a predictor, but it was collinear with population ID (VIF > 3) and hence omitted from the final analysis. To account for the potential role of selective disappearance on life-history traits correlated with life span, we fitted another model with the same set of variables but added life span as a covariate (Hayward et al., 2013). To ensure that the observed effect was not related only to early-life fecundity, we fitted another model with the above-mentioned covariates but with the sum of three consecutive clutches at the age when fecundity peaked in each population (WT 22–25 weeks, GRZ 9–11 weeks) instead of the first three clutches.

To test for a trade-off between early and late fecundity within biological age, we used linear regression with residuals corrected for body size and repeated measurements from a Negative binomial GLMM. We fitted separate GLMMs for early fecundity (first two clutches) and late fecundity (last two clutches within individual female life span). Fecundity was the response variable and body size was the explanatory variable. Female ID was treated as a random factor. Because fecundity is very variable between two consecutive clutches from the same female, a single average value for each female (i.e. the average calculated from two residual values) was used for linear regression to test the relationship between early-life and late-life fecundity. We used the first and last two clutches (rather than three as in the other analyses), because many GRZ females did not produce six experimental clutches over their lifetimes and the use of three clutches would considerably reduce sample size in this strain. Females which produced less than four clutches within their life span ($N = 10$ GRZ, $N = 0$ WT) were excluded from this analysis.

2.5 | Demography

Because fish have a long period of post-maturity growth, body size is an important correlate of female fecundity (Barneche et al., 2018). Growth trajectories (from weekly measurements over the life span) were compared between populations using sex-specific Gaussian Generalized additive models (GAM) from the package MGCV v 1.8.28 (Wood, 2017). Body size was the response variable and age was the explanatory variable with basis dimension $k = 10$ (a method suitable for killifish growth analysis, García et al., 2019). Population was added as an explanatory factor and fish ID was treated as a random effect to account for repeated measurements. Only data to the age of death of the last GRZ (shorter-lived strain) fish were used for statistical tests, but the complete dataset was visualized in plots.

The population-specific age at maturity was modelled using a Gamma GLM, with individual age of sexual maturity (the age of first oviposition) for each female as the response variable and strain ID as an

explanatory variable. Size at the maturity was compared between populations with a Gaussian linear model. Fecundity and fertilization rate peaks were estimated from predicted GAM curves (Figure 1). Total reproductive output (sum of eggs over the entire life span) and individual maximum clutch size were modelled with a Gamma GLM. Population-specific maximum fertilization rate was modelled with a binomial GLM. An overview of all models and their structure is given in Table S1.

2.6 | Reproductive senescence from the perspective of chronological age

Using weekly measurements of individual fecundity, fertility and fertilization rate, strain-specific trajectories were analysed using a GAM. GAMs are not restricted to following a specific polynomial form and continuous variables are fitted with local smoothing functions (Wood, 2017). Fecundity and fertility age-dependent trajectories were modelled with a negative binomial GAM, with the basis dimension chosen by comparison of AIC and constrained to a maximum of $k = 7$ for a smoother fitted to age to retain a biologically meaningful trajectory. Female body size was not used as a covariate because we were specifically interested in the effects of age, and female size was not a population-specific trait (see Figure S1 in Supporting Information). A similar approach was used for fertilization rate (raw binomial data: fertilized and unfertilized eggs), using a binomial GAM (WT $k = 7$, GRZ, $k = 5$, selected on the basis of AIC). Individual female identity was added as a random factor (specified as *bs = 're'*) to account for repeated measurements in fecundity, fertility and fertilization rate models.

We investigated whether decline in fertilization rate was caused by senescence in egg quality (female effect), senescence in sperm quality (male effect) or their combination by pairing young (8 weeks) and old (31 weeks, when a consistently lower fertilization rate was detected) males and females of the WT strain. To analyse these data, a binomial Generalized mixed effect models from the package LME4 v 1.1.21 (Bates et al., 2015) were used. Raw binomial data on egg fertilization were the response variable and the interaction between female and male age (factors with two levels) was the explanatory variable. Individual female and male identity were used as random factors to account for repeated measurements (for the overview of model structures, see Table S1).

2.7 | Reproductive senescence from the perspective of biological age

Individuals age at different rates and, hence, senescence patterns are often more apparent at biological rather than chronological age (Froy et al., 2019; Hayward et al., 2013). In the absence of environmental sources of mortality in our setting and the lack of physiological markers of biological age, we standardized biological age by using the time to death (i.e. weeks to death, age at death = 0) of individuals as an explanatory variable. Separate models with biological age were fitted for each population due to population-specific longevity. Fecundity was fitted using a negative binomial GAM, weeks to death had basis dimension $k = 7$ for WT and $k = 5$ for GRZ. Fertilization rate was fitted using a binomial GAM (WT: $k = 6$, GRZ: $k = 7$). Female ID was treated as a random factor in the models. All basis dimensions (k) were selected by comparing the AIC with the upper limit restricted to $k = 7$ to retain a biologically meaningful trajectory. Finally, we compared models of chronological and biological age using AIC to test which measure of age has stronger explanatory power.

To determine if change in terminal (i.e. 30 days prior death) reproductive function differed between long-lived and short-lived females, we split our data by those with life spans above and those with life spans below the population-specific median. The last 30 days were chosen arbitrarily because this period provided a roughly equal sample size (4–5 bouts per female) for short- and long-lived individuals of both populations. Terminal fecundity was modelled using a negative binomial GAM with a population- and longevity-specific relationship (i.e. factor with four levels) to days to death and basis dimension $k = 3$. Body size was used as a covariate to account for differences in body sizes of females that died earlier and later. Female ID was a random factor. Terminal fertilization rate was modelled using a binomial GAM with $k = 5$ with a population longevity-specific relationship to days to death. Female ID was used as a random factor. The basis dimension was always chosen by AIC. An overview of all models related to biological age and their structure is presented in Table S2.

3 | RESULTS

3.1 | Demography

Weekly measurements of body size demonstrated that outbred WT and inbred GRZ females did not differed significantly in their body size (Gaussian GAM, $F_{1,847} = 3.14$, $p = 0.077$, Figure S1). Growth was asymptotic and females of both strains effectively ceased growth at an age of 12–14 weeks (Figure S2), several weeks after sexual maturation (Table 1). Despite matching body size, WT females greatly outperformed GRZ females in all fitness-related characteristics—longer life span (Figure 1a), earlier maturity, smaller body size at maturity, higher fecundity and higher fertilization rate (Table 1; Tables S3–S5).

3.2 | Reproductive senescence appeared long after cessation of growth

Fecundity increased from sexual maturity until a prime age and then declined (Figure 1b). Peak fecundity was achieved 9 weeks after growth cessation in WT females (at the age of 23 weeks), but occurred earlier and corresponded with growth cessation in GRZ females (age 13 weeks). These ages correspond to 50% survival of females in both populations (Figure 1a). Age-dependent fertility was high since cessation of growth until the age of 25 weeks when fertility declined in both population (Figure 1c). Fertilization

Life-history trait	Wild-type (WT)	Laboratory (GRZ)	Test statistics
Median life span (weeks)	23	13	log-rank test, $\chi^2_1 = 19.3$, $p < 0.001$
Age at maturity (weeks)	5.9 ± 0.13	8.3 ± 0.21	Gamma GLM, $\chi^2_1 = 108.9$, $p < 0.001$
Body size at maturity (mm)	28 ± 0.4	32 ± 0.5	Gaussian LM, $F_{1,57} = 35.29$, $p < 0.001$
Most fecund clutch size (*N* eggs)	92 ± 13	34 ± 6	Gamma GLM, $\chi^2_1 = 18.93$, $p < 0.001$
Lifetime fecundity (*N* eggs)	668 ± 146	197 ± 50	Gamma GLM, $\chi^2_1 = 11.92$, $p < 0.001$
Max. fertilization rate (%)	90 ± 5.2	64 ± 9.9	Binomial GLM, $\chi^2_1 = 5.76$, $p < 0.001$

TABLE 1 Life-history traits of females from two populations of *Nothobranchius furzeri* differing in number of captive generations. Detailed test statistics are presented in Supporting Information (Tables S1–S3)

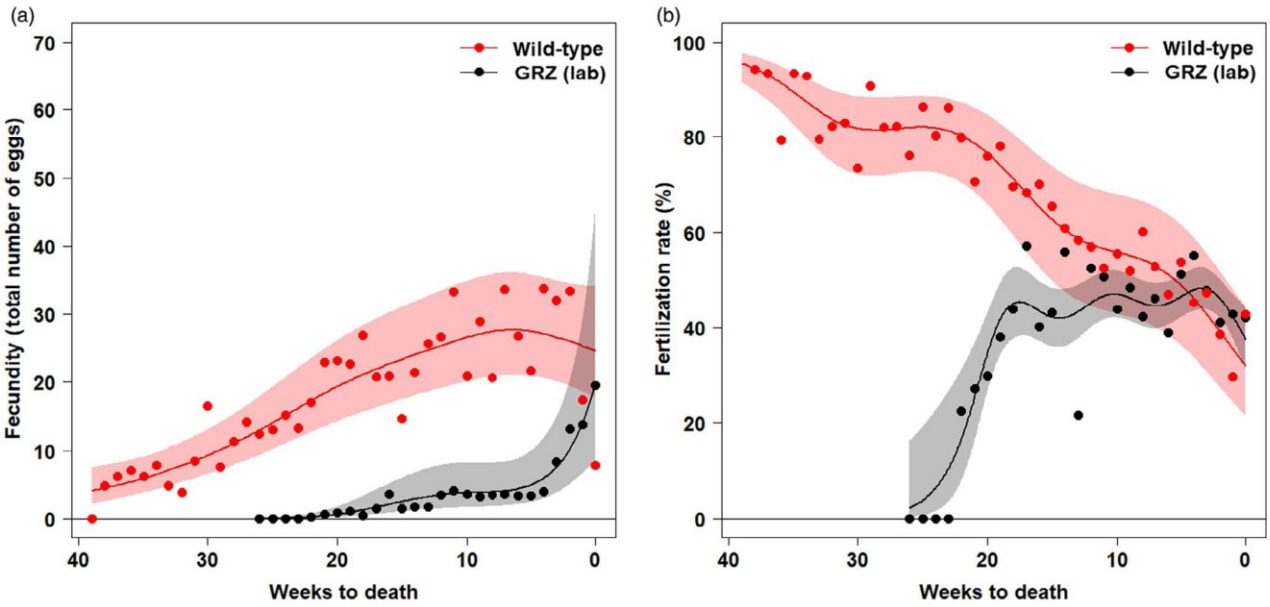


FIGURE 2 Fecundity (a) and fertilization rate (b) assessed on biological age (weeks to death) of individual survival in wild-type (WT) and laboratory (GRZ) populations. WT population is red and GRZ population is black. Data are modelled with a negative binomial GAM for fecundity and binomial GAM for fertilization rate. Shaded area represents 95% confidence interval. Points are means generated from GLMM (negative binomial for fecundity and binomial for fertilization rate) accounted for repeated measures of each individual and with weeks before death as a factor

rate peaked later than fecundity in the GRZ population (21 weeks) but at a similar age as peak in fecundity in WT (Figure 1d) and its decline coincided with the decline in fecundity in WT females (Figure 1d).

3.3 | Reproductive senescence from the perspective of biological age

In contrast to chronological age, there was only weak senescence in fecundity for biological age in WT (NegBin GAM, *edf* [effective degrees of freedom] = 3.189, $\chi^2 = 77.78$, $p < 0.001$, Figure 2a) and increase in fecundity was apparent in GRZ (*edf* = 3.325, $\chi^2 = 59.7$, $p < 0.001$, Figure 2a). However, the fecundity–age relationship had a clearly superior fit when chronological age was used (WT: ΔAIC = 118, GRZ: ΔAIC = 232, Table S6). Biological-age senescence in fertilization rate was apparent in WT population where it declined prior to death (*edf* = 4.96, $\chi^2 = 868.6$, $p < 0.001$, Figure 2b) but was relatively stable after reaching an asymptote in GRZ (*edf* = 5.938, $\chi^2 = 1{,}142.0$, $p < 0.001$, Figure 2b). Again, models with chronological-age senescence in fertilization rate had a superior fit over the biological-age senescence model (WT: ΔAIC = 368, GRZ: ΔAIC = 50, Table S6).

After splitting females into long- and short-lived groups, there was a statistically significant terminal decrease in fecundity only in long-lived WT individuals (*edf* = 1.235, *F* = 17.208, $p < 0.001$,

Figure S4) and a non-significant tendency towards a decrease in fertilization rate prior to death in long-lived WT females ($p = 0.082$) but no detectable terminal decrease in fecundity and fertilization rate in any other group ($p > 0.271$, Figure S4).

3.4 | Faster functional reproductive senescence in females

Senescence in fertilization rate depended on both sexes (binomial GLMM, female.age:male.age interaction, $\chi^2_1 = 3.97$, $p = 0.0463$, Figure 3) but there was a much stronger effect of female age ($\chi^2_1 = 22.72$, $p < 0.001$) than male age ($\chi^2_1 = 2.22$, $p = 0.136$). The fertilization rate in old females was low irrespective of male partner age (12.1 ± 5.0% [mean ± *SE*] with old males; 12.4 ± 4.9% with young males) but young female success was higher with young males (69 ± 8.7%) than with old males (52.7 ± 10.1%, Figure 3).

3.5 | Positive association between reproduction and life span

Female life span was positively associated with early-life fecundity (calculated as the sum of eggs in the first three clutches, $p = 0.002$, Table 2) and there was no association between life span and juvenile growth (measured as body size at the age of 8 weeks: $p = 0.707$, Table 2) or life span and maximum fertilization rate ($p = 0.540$, Table 2). Similar relationships were observed when the model was fitted to the population-specific age of peak fecundity (i.e. mid-adulthood, peak fecundity: $p = 0.006$, Table S7) and when it accounted for selective disappearance (early fecundity: $p = 0.001$, Table S8). Contrary to the assumption of reproduction trade-off, there was a tendency towards a positive relationship between early-life and late-life fecundity (linear regression of residuals from GLMM corrected on body size and repeated measures, $F_{1,48} = 3.08$, $p = 0.085$, Figure S5).

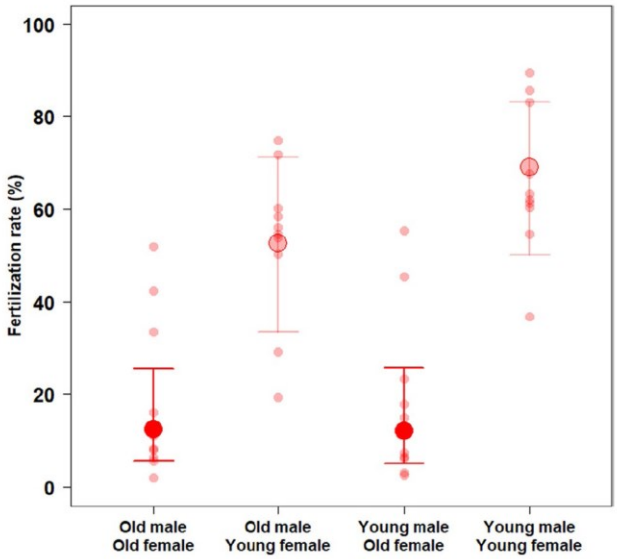


FIGURE 3 Decrease of fertilization rate of 31-week females but not old males of the wild-type population. Large points are means, error bars are 95% confidence intervals. Dark-red points are means for old female combinations. Light-red point means are for young female combinations

TABLE 2 Summary table of the Gamma GLM for predictors of female life span in *Nothobranchius furzeri*. Life span data were square-root transformed. All explanatory variables (except 'Population') were zero-centred with *SD* = 1 prior to analysis

	Estimate (*SE*)	*t*-value	*p*
Intercept	5.15 (0.071)	72.4	**<0.001**
Number of eggs in the first three clutches	0.16 (0.050)	3.2	**0.002**
Maximum fertilization rate	0.05 (0.059)	0.8	0.429
Body size at 8 weeks	−0.03 (0.053)	−0.5	0.625
Population (laboratory)	−0.50 (0.124)	−4.3	**<0.001**

4 | DISCUSSION

Reproductive senescence has long been considered absent in fish, given their post-maturity growth and strong increase in fecundity as a function of increasing body mass (Barneche et al., 2018; Bidder, 1932; Finch, 1990). We report a clear case of reproductive senescence in a fish with a long post-maturation growth period, unusually rapid development and short life span. We show that the pattern of reproductive senescence was sex-dependent, varied between populations with a different history of captive breeding and genetic diversity. Reproductive senescence was stronger in females than in males. Individual life span was positively associated with fecundity and unrelated to juvenile growth, not supporting the role of allocation trade-offs in reproductive senescence.

Our longitudinal study had greater power to detect reproductive declines than previous cross-sectional studies on senescence in killifish (Blažek et al., 2017; Vrtílek, Žák, Blažek, et al., 2018) because individual-based longitudinal studies provide more accurate senescence estimates (Nussey et al., 2008; Warner et al., 2016). Fish and other animals with indeterminate and post-maturity growth have long been considered insusceptible to ageing, but recent studies demonstrate that ageing may be common in these species (Benoît et al., 2018; Depeux et al., 2020; Warner et al., 2016). Indeed, other fish species are reported to senesce, including anchovies (Parrish et al., 1986), guppies (Reznick et al., 2004) and herring (Benoît et al., 2018). Reproductive senescence among species with a long period of post-maturity growth does not appear to be restricted to those with rapid life history but rather to the availability of longitudinal data. For example, long-lived painted turtles, considered to lack senescence, were revealed to senesce in the wild, though the onset of senescence was postponed compared to animals that cease growing soon after sexual maturation (Warner et al., 2016). Limited

knowledge of reproductive senescence in some organisms, including model organisms, prevents a solid understanding of the mechanisms related to decline in this key fitness component (Lemaitre et al., 2020).

Low effective population size leads to the accumulation of mutation load (Cui et al., 2019). Inbreeding, an extreme version of limited effective population size, affects reproductive traits (DeRose & Roff, 1999). WT females matured at smaller size than GRZ females but maintained similar fecundity of their first batches as GRZ females. Maturing at small size and maintaining high fecundity may be beneficial to life in time-constrained environment such as ephemeral savanna pool inhabited by annual killifish. Given that captive environment is not time-constrained, delayed maturity in GRZ may arise from adaption to captivity.

Laboratory populations are selected for successful reproduction in captivity and, hence, are often more fecund (despite the cost of inbreeding) than WT strains under matching captivity conditions (Miller et al., 2002; Sgro & Partridge, 2000). A shorter life span is often linked to accelerated reproduction (Austad & Hoffman, 2018; Charnov et al., 2001; Miller et al., 2002). Short life span in GRZ may be associated with a high mutation load (Willemsen et al., 2020), which is supported by the fact that GRZ crosses with a WT population had an intermediate life span (Kirschner et al., 2012). Laboratory GRZ had a lower maximum clutch size, but their fertility and fecundity until the onset of senescence were similar to the WT population. It appears that population-specific fecundity may simply arise from an earlier onset of reproductive senescence in GRZ.

There are contrasting predictions for reproductive senescence between a fast life history and post-maturity growth. Evolutionary theories of ageing (Finch, 1990; Vaupel et al., 2004; Williams, 1957) predict that organisms with long post-maturity (or indeterminate) growth experience delayed senescence, or its absence, as a consequence of increased fitness of larger/older individuals. In contrast, short-lived organisms with high adult mortality are expected to evolve an early onset of senescence (Jones et al., 2008). We established that reproductive senescence in a short-lived species with post-maturity growth started much later than a deceleration in growth. Growth rate in organisms with a long post-maturity growth period is asymptotic rather than linear and decelerates at older age (Sebens, 1987; Warner et al., 2016). The prime age for fecundity coincided with 50% female survival and the consequent decline in fecundity suggests its association with somatic deterioration (Ricklefs et al., 2003). Comparing the onset of reproductive senescence with constraints imposed on annual killifish by the persistence of their natural habitats (Vrtílek, Žák, Polačik, et al., 2018) showed that senescence coincided with the time when only a few individuals would be expected to survive in rapidly desiccating pools. This correspondence of senescence with disappearance of the habitat provides the opportunity for late-acting detrimental mutations to be fixed in the population (Cui et al., 2019; Medawar, 1952). Reproductive senescence has, therefore, limited impact in natural populations (Vrtílek, Žák, Blažek, et al., 2018).

Functional reproductive senescence was greater in females. We detected a much lower reduction in fertilization capacity in old males than in old females. The observed decline in fertilization rate of eggs was mainly the result of a decreased capacity of females to produce viable eggs. This outcome is surprising as male killifish suffer higher mortality in the wild (Reichard et al., 2014) and twice as many histopathological lesions were reported in testicular in comparison with ovarian tissue under captive conditions (Di Cicco et al., 2011), leading to the prediction for earlier onset of reproductive senescence in males (Bonduriansky et al., 2008; Di Cicco et al., 2011). A weak association between gonad histopathology and reproductive function is puzzling, but has previously been observed in male southern platyfish *Xiphophorus maculatus* (Schreibman et al., 1983). A caveat is that we did not test spermatozoa traits or juvenile survival (Arslan et al., 2017), though our experimental data clearly demonstrate that older males were fully capable of fertilizing eggs.

Biological age can often describe senescent patterns more reliably than chronological age, as it controls for selective disappearance of less fit individuals from the population with time (Froy et al., 2019; Hämäläinen et al., 2014; Hayward et al., 2013). On the scale of biological age (weeks to death), we detected only a slight terminal decrease in fecundity in WT population while fecundity actually increased with biological age in GRZ. The fecundity pattern observed in GRZ may be interpreted as a terminal investment (e.g. Froy et al., 2013). However, we believe rather that it is related to the contribution of short-lived individuals to terminal population-specific biological age, having died at a chronological age when their fecundity was still steeply increasing. In WT fish, reproductive senescence in fertilization rate was detected since it declined throughout biological age. In contrast, in the GRZ population, reproductive senescence in fertilization rate was asymptotic. Despite this insight into reproductive senescence from the perspective of biological age, the observed age-related patterns of reproductive function in *N. furzeri* were better explained by models of chronological age. Terminal reproductive senescence was a feature of long-lived females because no detectable decline was observed in short-lived females (Figure S4) which corroborates the conclusion that chronological age was more important.

We speculate that the difference between GRZ and WT in reproductive senescence at biological age is related to the relatively premature death of GRZ fish, corresponding with a lower expression of phenotypic markers of ageing in the GRZ strain compared to wild-derived populations (Terzibasi et al., 2008; Valdesalici & Cellerino, 2003). Indeed, only 16% of GRZ fish survived to an advanced age when a decline in fertilization rate (from the perspective of chronological age) emerged, contrasting with 50% survival of WT females at the age when their fertilization rate started to decline.

A positive association between early reproduction and life span, and a positive association between early and late reproduction are incongruent with reproduction–life span trade-offs (Kirkwood, 1977; Stearns, 1992). High allocation into early reproduction is likely under strong selection in annual killifish, since

natural pools can disappear in <3 weeks after initial inundation (Vrtílek, Žák, Polačik, et al., 2018). While we acknowledge that captive conditions are not ideal for testing life-history trade-offs given the absence of resource limitation in captivity (Ricklefs & Cadena, 2007), we can discount the confounding effects of a novel laboratory environment (Service & Rose, 1985) and inbreeding (Rose, 1984). Our study populations differed in both these aspects, but retained the same pattern of positive association between early reproduction and life span. Silver spoon effect may play the role in observed pattern because it predicts that favourable conditions (such as captivity conditions) early in life have a long-term positive effect on fitness (Grafen, 1988). Additionally, the shorter life span and lower fecundity of GRZ compared to WT fish is congruent with results reported for *Drosophila melanogaster*, where mutations decrease early adult fitness through reduced life span (Kimber & Chippindale, 2013). This observation would support positive pleiotropy (Maklakov et al., 2015). We acknowledge that our data cannot distinguish among those concepts and further experimental research is needed to disentangle mechanisms behind positive correlation of phenotypic traits in annual killifish.

We estimated senescence in a limited number of reproductive traits and focused mainly on female traits. The focus on female reproductive senescence in our study was driven by the impossibility of extracting *N. furzeri* sperm non-invasively (Dolfi et al., 2020). Reproductive senescence in males was negligible but we acknowledge that other male reproductive traits such as sperm traits, ornaments (Cornwallis et al., 2014) or offspring performance (Preston et al., 2015), unmeasured in our study, can be subject to reproductive senescence. Following offspring survival and performance beyond 24 hr post-fertilization was not feasible during our study, especially given the number of eggs we analysed (N = 12,480 fertilized eggs) and developmental variability of diapausing killifish eggs (Polačik et al., 2014). A dedicated longitudinal study may provide insights into the effect of parental reproductive senescence on offspring performance.

In conclusion, we demonstrate strong reproductive senescence in a short-lived annual fish, a vertebrate with a long post-maturation growth period. It remains to be tested how common reproductive senescence is among fish, as well as other species with post-maturity and indeterminate growth and diverse life histories, including long-lived species. These data would illustrate the evolutionary constraints on reproductive function and fitness.

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AUTHORS' CONTRIBUTIONS

J.Ž. and M.R. designed the study and wrote the paper; J.Ž. performed the research and analysed the data.

DATA AVAILABILITY STATEMENT

Data supporting the results and the R codes of all our analyses are available from Figshare Repository https://doi.org/10.6084/m9.figshare.12095961 (Žák & Reichard, 2020).

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PAPER 7: Diel pattern in activity of the wild *Nothobranchius* fishes

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Diel schedules of locomotor, reproductive and feeding activity in wild populations of African annual killifish

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Diel patterns of different activities arise from adaptations to periodic cycling of environmental parameters and may involve trade-offs between acquiring benefits and minimizing associated costs. In this study, we provide fundamental baseline data on diel activity of natural populations of *Nothobranchius* fishes, model organisms in laboratory studies, including links between diurnal rhythms and ageing. Initially, we quantified the diel change in activity in wild populations of three African killifish species (*Nothobranchius furzeri*, *Nothobranchius orthonotus* and *Nothobranchius pienaari*) and compared average activity between sexes. In all species, males were more active than females, probably as a result of their active pursuit of females. Swimming activity peaked at midday. In *N. furzeri*, the only species occurring at all sites, oocytes were ovulated in the early morning, and most spawning events had occurred by the early afternoon. Gut fullness and diet richness increased before spawning activity and peaked in the morning. Daytime diet was dominated by chironomid larvae, whereas notonectid bugs were the dominant prey at night, perhaps as a result of different prey detectability over the diel cycle. Finally, no loyalty to any particular pool section was detected in *N. furzeri*. Collectively, these data provide the first empirical description of diel activity in three wild populations of African killifish.

ADDITIONAL KEYWORDS: activity pattern – circadian activity – *Nothobranchius*.

INTRODUCTION

Organisms face periodic environmental changes, ranging from seasonal to diel cycles (Beale *et al.*, 2016). In animals, adaptation to the periodicity of 24 h cycles (diurnal rhythms) involves conspicuous diel patterns of particular activities, including individual locomotor activity (Davis, 1964), feeding regime (Metcalfe *et al.*, 1999) and reproductive behaviour (Lema *et al.*, 2010). Physiologically, diurnal rhythms are synchronized endogenously (Ekström & Meissl, 1997). From an ecological and evolutionary perspective, diurnal rhythms have evolved to match particular activities with the most suitable abiotic (e.g. light intensity, temperature, oxygen level) and biotic (food availability, reproduction, intraspecific competition or predation risk) parameters (Daan, 1981; Helfman, 1986). For example, patterns of locomotor activity are optimized to allocate energy effectively for finding food, finding mating partners and rest (Werner *et al.*, 1993; Smith & Blumstein, 2008) and represent a compromise between particular costs (e.g. predation risk, energy expenditure) and benefits (e.g. finding food or a partner) (Werner *et al.*, 1993; Metcalfe *et al.*, 1999).

The cost–benefit trade-offs behind temporal dynamics in activity patterns are often sex specific (Perrin & Mazalov, 2000; Croft *et al.*, 2003). Females allocate more energy to gamete production (Trivers, 1972; Graf *et al.*, 2010), whereas males are often the more active sex, being involved in searching for and competing for access to mates (Haas, 1976a; Croft *et al.*, 2003). Behavioural trade-offs constrain the concurrent incidence of two behaviours (Cuthill & Houston, 1997) and result in diel periodicity of specific behavioural repertoires, particularly in organisms with daily reproductive activities. For example, in Amargosa pupfish, *Cyprinodon nevadensis amargosae* Miller, 1948, feeding activity peaks in the morning, whereas courtship and spawning activity peaks in the afternoon (Lema *et al.*, 2010). Laboratory experiments have demonstrated that reproductive cycles are largely controlled endogenously and are not directly

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dependent on timing of the feeding regime (Weber & Spieler, 1987).

The main goal of this study was to characterize the diel change of locomotor activity, reproduction and feeding in wild populations of African annual killifishes of the genus *Nothobranchius*. In controlled laboratory conditions, *Nothobranchius* fishes are primarily active during daylight (Lucas-Sánchez *et al.*, 2013; Almaida-Pagán *et al.*, 2018), and light conditions are probably important in mate searching. Males are larger and brightly coloured (Sedláček *et al.*, 2014) and actively pursue females (Polačik & Reichard, 2011). Active mate searching indicates the importance of locomotory activity in male reproductive success. However, their higher activity and bright coloration expose males to higher predation risk from ambush predators (Haas, 1976b), which might explain why wild populations tend to be female biased (Reichard *et al.*, 2014). Light conditions might affect the feeding activity because *Nothobranchius* spp. are visual micropredators; their brain anatomy is characterized by a prominent optic tectum with small olfactory bulbs (D'Angelo, 2013), and their diet contains a large proportion of small, actively moving aquatic invertebrates (Polačik & Reichard, 2010). Thus, light conditions might have a profound effect on locomotory activity.

In addition to the main goal, we tested their loyalty to particular sites, suggesting potential territoriality. Territorial behaviour could affect the activity of an individual owing to its spatial restriction (Hanson *et al.*, 2008). Male territoriality is common in fishes, especially in sexually dimorphic species where males are larger (Gross & Sargent, 1985; Kodric-Brown, 1988). In laboratory conditions, male *Nothobranchius* have been observed to defend a spot with a spawning substrate (Polačik *et al.*, 2016). Although this has been implicated indirectly for wild populations (Reichard *et al.*, 2009), no study has yet examined territoriality of *Nothobranchius* males in the wild.

Nothobranchius fishes have an extremely rapid life history adapted to life in ephemeral pools (Reichard & Polačik, 2019). The eggs survive desiccation in the substrate, and fish hatch after heavy seasonal rains that briefly inundate their habitat. Upon hatching, they grow rapidly and mature within 2 weeks (Vrtílek *et al.*, 2018b). Sexually mature females ovulate a batch of 20–120 eggs every day (Vrtílek & Reichard, 2016). This represents high energetic demands that need to be covered by effective feeding. Under strong pressure of habitat desiccation (Grégoir *et al.*, 2018), they devote a significant proportion of time to reproduction. This might lead to behavioural conflict between feeding and reproduction, potentially resulting in temporally synchronized schedules of feeding and reproductive activity to maximize their efficiency.

We estimated the diel change of locomotor activity in wild populations of three species of African annual killifish. *Nothobranchius furzeri* Jubb, 1971, *Nothobranchius orthonotus* (Peters, 1844) and *Nothobranchius pienaari* Shidlovskiy *et al.*, 2010, in their natural habitat in southern Mozambique. We predicted high locomotor activity throughout the day, associated with feeding in the morning, when fish have to replenish energy reserves after the nocturnal period, and reproductive activity prevailing later in day after egg ovulation (Haas, 1976a; Lema *et al.*, 2010). This would be indicated by temporal dynamics in gut fullness, gonad mass and the number of ovulated oocytes in female ovaries. We further predicted males to be more active than females on average, driven by their reproductive strategy of active mate searching. Finally, we investigated territoriality in wild *Nothobranchius* by examining the fidelity of individual fish to a particular section of the pool using a spatially informed capture–mark–recapture study. Overall, this study provides the first evidence of diel change of locomotor activity, feeding regime and reproduction in wild *Nothobranchius* populations. This is important because African annual killifish are an established laboratory model in biomedical and ecological research (Cellerino *et al.*, 2016; Hu & Brunet, 2018), including circadian rhythmicity with regard to senescence (Valenzano *et al.*, 2006; Terzibasi Tozzini *et al.*, 2017).

MATERIAL AND METHODS

The study was conducted at four sites (ephemeral pools in southern Mozambique) between March and May 2016. An overview and details of the sampling sites and procedures are given in Table 1, and detailed information on sample sizes is presented in the Supporting Information (Table S1).

Diel change of activity

To estimate diel change of fish activity, a 24 h snapshot study at three sites (sites 1–3) was performed (Table 1). Fish activity was estimated using non-baited minnow traps (260 mm × 260 mm × 450 mm, with two 55 mm funnel openings, mesh size 4 mm; Zebco Europe, Tostedt, Germany), a passive sampling method that required fish movement for capture (Diaz Pauli *et al.*, 2015; Žák *et al.*, 2018a). A pilot study showed that baited and non-baited traps were equally efficient for killifish, and non-baited traps did not attract lungfish, a potential killifish predator (Reichard *et al.*, 2014). No other fish species occurred at the study sites. Four (site 1) or six (sites 2 and 3) traps were used, depending on pool size and fish density. The traps were

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Table 1. Overview of sampling sites

Site	GPS	Analyses	Trapping	Reference sampling	Fish age (days)	Pool area (m^2)	Maximal depth (cm)	Number of traps	CPUE (individuals per haul) of species ratio F:O:P and sex ratio F:M | F:M | F:M
Site 1	22°30′28″S 32°34′54″E	Activity	22 March	22 March	56	220	20	4	12.5:54.0:0.5
Site 2	23°30′15″S 32°34′21″E	Activity, dispersal, diet and reproductive parameters	30 April	1 May	50	1265	110	6	9.5:3.0 | 36.0:18.0 | NA 3.0:0.8:0.0 2.4:0.6 | NA | NA
Site 3	24°18′15″S 32°36′55″E	Activity	16 March	17 March	78	760	35	6	2.0:2.0:5.8 NA | NA | 5.8:0.0
Site 4	23°38′42″S 32°36′07″E	Reproductive parameters	NA	6 May	53	1110	45	–	5.0:0.2:0.4 3.8:1.2 | NA | NA

Catch per unit effort (CPUE) of species and sex ratio represent the number of fish per one seine net haul. Species and sex ratios were estimated from seine net samples (site 1, two seine net hauls; site 2, five seine net hauls; site 3, five seine net hauls) Owing to dense vegetation at site 3, additional dip net samples were taken to obtain more appropriate species and sex ratios, which differ slightly from the presented values from seining. Fish age is taken from Vrtílek *et al.*, (2018a) and was estimated from otolith readings (Reichard *et al.*, 2017) and recalculated for the date of reference sampling at a particular site.

Abbreviations: F, *Nothobranchius furzeri*; F:M, female-to-male ratio, which is presented only for species and sites where sex-specific activity was analysed (sample size greater than five); O, *Nothobranchius orthonotus*; P, *Nothobranchius pienaari*.

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spaced (55–211 m^2 per trap) to minimize interference. All available pool habitats (deep water, surface water, vegetated sections and open sections) were sampled to capture potential non-random movement of fish in the pool. Trap openings were always fully submerged. Trapping over 24 h yielded 234 fish of three species across all three study sites (Supporting Information, Table S1).

During the day, traps were checked every hour to prevent trap saturation (Prchalová *et al.*, 2011; Bacheler *et al.*, 2013). At night, traps were checked at 3 h intervals because there was a lower risk of trap saturation; laboratory studies have demonstrated that *Nothobranchius* fishes are more active during the day (Lucas-Sánchez *et al.*, 2011). Fish removed from traps were immediately identified to species. Males possess species-specific colour patterns, and females can be readily separated by body shape and mouth position (Wildekamp, 2004). Then, fish were immediately released at the place of capture. The probability of recapturing the same individual was likely to be very low; a trap sampling at site 2 where 237 colour-marked *N. furzeri* were released (in one of four colours; see 'Home range in *N. furzeri*' section), 15 colour-marked individuals were recaptured. With that set of four colours on 237 individuals, no fish with the same colour was recaptured in the same trap in two consecutive time periods. Five identical combinations (colour/sex/trap) across two (of six) traps used at that site were captured during 24 h trap sampling, but it is not likely that they represented the same individual fish.

Each trap was equipped with a waterproof temperature and light datalogger (HOBO Onset Computer, UA-002-64, Bourne, MA, USA). Data were recorded every 1 min. In each pool, surface and bottom temperatures were recorded (Supporting Information, Fig. S1). Light intensity (in lux) was collected from the water surface datalogger (Supporting Information, Fig. S2). Oxygen concentration (in milligrams per litre) was measured in close proximity to the traps with a YSI ProODO hand-held meter (YSI Inc./Xylem Inc, USA) at site 3. No oxygen meter was available for sampling at the other sites. One surface (5 cm below the water surface) and one bottom (10 cm above the substrate) measurement was completed near each trap (Supporting Information, Fig. S3). Oxygen measurements were completed before every second checking of each trap during the day and every 3 h overnight. The mean values calculated from three surface and three bottom measurements of oxygen concentration at each 2 h sampling interval were used for analysis (at night, they were estimated as the mean value from the two closest sampling points).

For statistical analysis, fish activity was expressed as the cumulative number of fish collected per 2 h of trapping relative to the total catch (sampling unit, altogether from three sites; N = 36 sampling units). A 2 h trapping interval was chosen to reduce the probability of zero captures during the period of fish activity. The three species were pooled in the analysis, given variations in environmental (e.g. size, turbidity, habitat structure) and biotic (species and sex ratio, fish density) conditions that confound a species-specific interpretation of activity. In addition, we obtained more adequate values for each sampling point by species pooling (N = 234 fish) and a better model fit indicated by the Akaike information criterion (AIC) (AIC pooled = 102; AIC with species identity = 216). All three coexisting species are also ecologically similar (Žák *et al.*, 2018b; Lambert *et al.*, 2019), which makes data pooling ecologically relevant. The data were square-root transformed, enabling the use of a generalized additive model (GAM) with a Gaussian error distribution. A GAM with a binomial error structure was originally used, but it yielded a skewed distribution of residuals and low model predictability.

All recorded explanatory variables were strongly associated and non-independent (concurvity 0.62–0.99). Therefore, separate models were fitted for each explanatory variable (always with and without an interaction with site), and their relative fits were compared using the AIC (Table 2) (Wood *et al.*, 2016). Given superior fits of models with interactions, the effect of predictor on site-specific activity was interpreted from interaction terms. All continuous variables (except for the response variable and time of day, given that sampling times were the same at all sites) were standardized (zero-mean centred and scaled to 1 SD) before analysis to enable interpretation of models with an interaction term (Schielzeth, 2010).

Sex-specific activity

Reference estimates of sex ratio were obtained using a seine net (2.7 m long, 0.7 m deep, mesh size 4 mm) and a dip net (triangular 0.45 m × 0.45 m × 0.45 m, attached to a 1.5 m wooden pole) within 1 day before or after trapping (Table 1). Seine and dip net collection provides unbiased estimates of killifish sex and species ratios (Diaz Pauli *et al.*, 2015; Vrtílek *et al.*, 2018a). Estimates were based on five seine net hauls at sites 2 and 3; site 1 accommodated only two hauls owing to its small surface area (Table 1). Site 3 was densely vegetated, making seine netting suboptimal; therefore, ratios were estimated from an additional 100 dip net sweeps only. Overall, reference sampling yielded 187 fish (34 *N. furzeri*, 117 *N. orthonotus* and 36 *N. pienaari*).

The hypothesis of higher male activity was tested by comparing sex ratios from traps with those from reference samples. For this analysis, samples were pooled across 24 h of trapping within each site.

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Table 2. Quantitative comparison of candidate models (generalized additive models with Gaussian error) of fish activity that included different environmental explanatory variables

Model name	Model structure	d.f.	ΔAIC
Light × Site	relative_catch~s(light,by=site)+site	13.009	0
Time × Site	relative_catch~s(time,by=site)+site	14.462	3.27
Light + Site	relative_catch~s(light)+site	12.839	15.3
Surface temperature × Site	relative_catch~s(surface_temperature,by=site)+site	9.081	34.42
Time + Site	relative_catch~s(time)+site	7.809	36.66
Bottom temperature × Site	relative_catch~s(bottom_temperature,by=site)+site	9.783	46.48
Surface temperature + Site	relative_catch~s(surface_temperature)+site	5	49.6
Bottom temperature + Site	relative_catch~s(bottom_temperature)+site	5	55.09

Degrees of freedom and difference in Akaike information criterion (ΔAIC) from the best model are shown for models with time of the day, light intensity, surface temperature and bottom temperature as explanatory variables, with and without an interaction between environmental predictor and site.

Only samples greater than five (species/sex/method combination) were used in the analysis. A generalized mixed-effect model (GLMM) with a binomial error distribution was used, with sex ratio as the response variable and sampling method (traps, reference/nets) as the explanatory variable. Species and site were included as random factors. A second model was fitted to test for a potential effect of species on sex-specific activity, with species as a fixed factor in an interaction with sampling method. Non-significant terms were removed from the model on the basis of AIC.

Home range in *N. furzeri*

One week before the activity experiment (Table 1), we estimated how far killifish disperse within site 2. We divided the pool area into four equal sections. In each section, killifish were collected using nine seine net hauls (5–10 m long). A total of 237 *N. furzeri* individuals were captured across all sections. Fish were anaesthetized with clove oil, measured for body size and marked subcutaneously on their upper flank with a section-specific VIE (visible implant elastomer; Northwest Marine Technology, USA) colour mark (33 green, 40 orange, 33 white and 131 yellow). After full recovery from anaesthesia, the fish were released back to the section of their origin. All sections were resampled after 24 h using the same method and effort. The VIE mark, sex and body size were recorded for all recaptured fish. We assumed that 1 week was sufficient for full recovery and did not affect the activity measurements (trapping) 1 week later.

Fidelity of *N. furzeri* to a particular section of the pool was estimated using a set of section-specific multinomial regressions. The proportion of individuals with a particular colour mark recaptured in a section were compared with 1000 sets of individuals randomly selected from all marked fish. The number of individuals in the random sample was equal to the number of individuals recaptured in the section. Predictors were origin (recaptured or randomly selected individuals), sex and body size. Confidence intervals (95%) of χ^2, *P*-values and likelihood of *P*-value being > 0.05, derived from 1000 permutations, were estimated for each section separately.

Diel pattern in feeding

Gut fullness, diet richness and diet composition were estimated from a sample of 100 *N. furzeri* (50 males and 50 females) collected at site 2. Five samples were taken at 05.00 (1 h before onset of activity), 10.30 (increasing activity), 14.30 (culminating activity), 17.45 (decreasing activity) and 00.00 h (zero activity) using a 2.7 m seine net until ten females and ten males were collected. Captured fish were euthanized with an overdose of clove oil and stored in 4% formaldehyde solution for later dissection. The fish were dissected in the laboratory at the Institute of Vertebrate Biology in Brno, Czech Republic. Their gut length was measured to the nearest millimetre. Gut fullness was estimated as the difference in mass between the full and rinsed gut (weighed to the nearest 0.001 g). Analysis of diet was conducted semi-quantitatively, by visual estimatiion of the relative biomass proportions of diet categories (De Crespin De Billy *et al.*, 2000). Fourteen categories were distinguished (Chironomidae, Notonectidae, Cladocera, Ostracoda, Copepoda, Odonata, Ephemeroptera, terrestrial insects, Coleoptera, Isopoda, tadpoles, killifish eggs, mineral matter and plant detritus). Diet richness was estimated as the number of different diet categories in the gut of individual fish.

The temporal pattern in gut fullness was analysed using GAM with a Gaussian error distribution. Gut fullness (mass of gut content) was the response variable, and time of day was a continuous explanatory variable (five sampling points) with a set basis dimension, k (k = 4 was selected over k = 5 on the basis of model

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parsimony based on equal AIC). Sex was added as a fixed factor, and gut length was included as a covariate ($k = 7$) because there was temporal change in gut length (ANOVA, $F_{4,94} = 5.24$, $P < 0.001$). Temporal dynamics in diet richness was tested using a GAM with a Poisson error (with unknown scale parameter = −1). Diet richness was the response variable, time of day was a continuous explanatory variable ($k = 4$), and sex was a fixed factor. Gut fullness was included as a covariate ($k = 3$).

Temporal dynamics in the probability of dominance of a specific diet category (i.e. diet category with the highest relative biomass in a particular individual) was examined using multinomial regression. Dominant diet category was the response variable, and time of day (a factor with five levels; factor was selected over continuous variable to observe a potential non-linear pattern, i.e. diel cyclicity), sex and their interaction were included as explanatory variables. Body size was added as a covariate. Multiple pairwise comparisons were performed using Tukey's HSD test.

Diel pattern in reproduction

Reproductive parameters (ovarian mass and the number of ovulated eggs in females and testicular mass in males) were estimated from the same individuals (i.e. 50 males and 50 females from site 2) as for dietary parameters and, additionally, from another 50 *N. furzeri* females from nearby site 4. Site 4 was sampled at five time points: 08.00, 10.00, 12.00, 14.00 and 17.00 h. Both sites were sampled on the same day (and were of comparable age; Table 1), 1 week after the diel activity change was estimated at site 2 (Table 1). The eviscerated body mass and gonad mass of each fish were weighed to 0.001 g. The number of ovulated oocytes (discernible by their orange translucent appearance) in ovaries was counted under a stereomicroscope.

The temporal pattern in gonad mass was analysed using sex and site (owing to site-specific sampling times) specific GAMs with a Gaussian error distribution. Changes in ovarian mass were tested using time of day as a continuous explanatory variable (both sites, $k = 5$) and eviscerated body mass as a covariate (site 2, $k = 4$; site 4, $k = 3$) given a superior estimate of model validity. Diel change in testicular mass at site 2 was analysed by linear regression (given the linear relationship), with time of day as a continuous explanatory variable and eviscerated body mass as a covariate. The site-specific change in the number of mature oocytes during the day was tested using zero-inflated Poisson GAM, with time of day as a continuous explanatory variable (site 2, $k = 5$; site 4, $k = 3$). Eviscerated body mass was not included as a covariate, because it did not vary temporally across sampling period (site 2, one-way ANOVA, $F_{4,45} = 0.57$, $P = 0.683$; site 4, $F_{4,45} = 2.12$, $P = 0.094$), and the number of oocytes was more affected by time of day than by female body mass (see Results). Note that adding eviscerated body mass to the model provided concordant conclusions.

All data analyses were conducted in the R v.3.4.3. environment (R Core Team, 2017), using *mgcv* (Wood, 2017), *MuMIn* (Bartoń, 2016), *lsmeans* (Lenth, 2016), *lme4* (Bates *et al.*, 2015) and *nnet* (Venables & Ripley, 2002) packages.

All work was carried out in accordance with relevant guidelines and regulations. Sample collection complied with the legal regulations of Mozambique (collection licence: ADNAP-170/7.10/16), and research procedures and experimental protocols were approved by the ethical committee of the Institute of Vertebrate Biology (79-17-UBO), in accordance with legal regulations of the Czech Republic.

RESULTS

Diel change in activity

A clear unimodal activity pattern was detected at two sites (sites 2 and 3), with the peak of activity at midday. No temporal change in diel activity was apparent at site 1 (Fig. 1; Table 3A). This variation among sites resulted in significant interactions between environmental predictors and site for all parameters measured (time of day, light level and water temperature; Table 2). Light level and time of day were the best predictors of diel activity change at sites 2 and 3 (Table 2), followed by water temperature. At site 3, where oxygen measurements were performed, killifish activity was also related to surface oxygen concentrations (Supporting Information, Table S1). Predictor-specific models are provided in Table 2.

Males were more active than females. The proportion of males in traps was 25% higher than in the reference sample (GLMM: $\chi^2 = 23.52$, d.f. = 1, $P < 0.001$; Fig. 2). Male-biased activity was consistent among all three species (GLMM: species, $\chi^2 = 3.43$, d.f. = 2, $P = 0.180$; species × method interaction, $\chi^2 = 2.54$, d.f. = 2, $P = 0.280$).

Home range

A total of 127 out of 237 marked *N. furzeri* were recaptured (54%). Recaptured fish were distributed randomly throughout the pool in relationship to their initial capture location, suggesting large home ranges. There was no effect of tag colour, sex or body size on the probability of recapture in the same pool section (Table 4).

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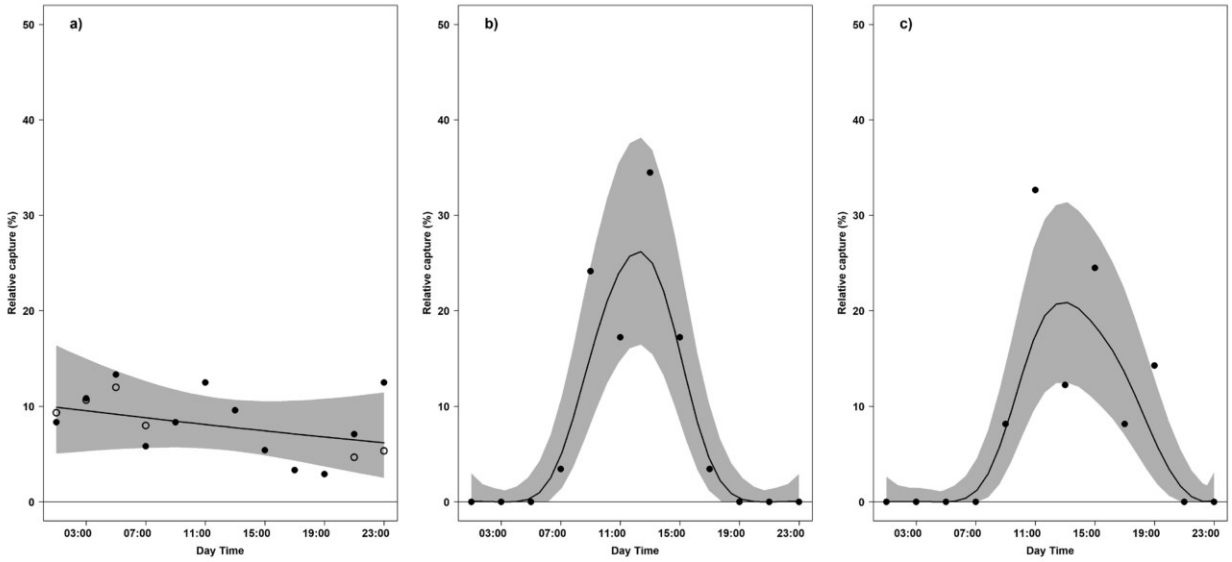


Figure 1. Diel change of locomotor activity. Diel dynamics of locomotor activity were estimated from the fit of generalized additive models at site 1 (A), with no peak in activity, and at sites 2 (B) and 3 (C), with a clear peak of activity. Filled circles represent relative catch per each sampling point calculated from the total catch. Open circles represent additional observations at site 1 (trapping continued after 24 h) that were not included in the analysis. Grey area indicates 95% confidence intervals.

Reproductive parameters

Ovarian mass declined significantly during the day at both sites [GAM: site 2, time $F = 6.50$, estimated degrees of freedom (e.d.f.) = 3.96, $P < 0.001$; site 4, time $F = 6.03$, e.d.f. = 3.751, $P < 0.001$], with a gradual decrease from noon (12.00 h) at site 4 and a mid-afternoon (14.30 h) decrease at site 2 (Fig. 3A). Eviscerated body mass was a significant covariate (site 2, $F = 5.82$, e.d.f. = 2.62, $P = 0.005$; site 4, $F = 17.26$, e.d.f. = 1.33, $P < 0.001$).

The number of ovulated oocytes had a clear unimodal pattern at both sites (GAM: site 2, $\chi^2 = 474.40$, e.d.f. = 3.95, $P < 0.001$; site 4, $\chi^2 = 113.5$, e.d.f. = 1.89, $P < 0.001$; Fig. 3B). Interestingly, peaks in the number of ovulated oocytes differed between the sites. At site 4 (sampled at 08.00, 10.00, 12.00, 14.00 and 17.00 h), the highest number of ovulated oocytes was recorded at 08.00 h, with all ovulated oocytes spent by 14.00 h. At site 2 (sampled at 05.00, 10.30, 14.30, 17.45 and 00.00 h), females had not ovulated oocytes 1 h before sunrise (05.00 h), and the model suggested that their number peaked between 10.30 and 14.30 h, followed by a sharp decline (Fig. 3B).

Testicular mass did not vary over the diel cycle ($F = 0.01$, d.f. = 1, $P = 0.944$), and eviscerated body mass was a significant covariate of testicular mass ($F = 25.84$, e.d.f. = 1.00, $P < 0.001$).

Diet and feeding patterns

Gut fullness varied significantly over time (GAM: time, $F = 6.83$, e.d.f. = 2.90, $P < 0.001$), being lowest before sunrise (05.00 h), peaking at 10.30 h and then gradually decreasing (Fig. 4A). Gut fullness did not differ between the sexes ($F = 2.14$, d.f. = 1, $P = 0.147$). Gut length was a significant covariate of gut fullness ($F = 7.91$, e.d.f. = 5.23, $P < 0.001$). The time × sex interaction was omitted from the final model (ΔAIC +4.28).

Diet richness also exhibited temporal dynamics (GAM: time, $F = 3.17$, e.d.f. = 2.86, $P = 0.030$), with a peak in mid-morning (10.30 h; Fig. 4B). Gut fullness ($F = 5.11$, e.d.f. = 1.34, $P = 0.021$) was a significant covariate. The sexes did not differ in diet richness ($F = 0.85$, d.f. = 1, $P = 0.359$). The time × sex interaction did not improve model fit (ΔAIC = −1.06) and was omitted from the final model. Within individuals, diet was dominated by chironomid larvae (40% of individuals), notonectids (35%), non-aquatic insects (17%) and Odonata larvae (5%). Copepoda, tadpoles and Ephemeroptera were rarely dominant (< 5% individuals), hence they were omitted from analysis of the diel pattern along with all minor diet categories (Cladocera, Ostracoda, Coleoptera, Isopoda, killifish eggs, mineral and plant detritus). The dominant diet varied over the day (multinomial regression: $\chi^2 = 88.2$, d.f. = 12, $P < 0.001$) and was significantly affected by fish body size ($\chi^2 = 11.3$, d.f. = 3, $P = 0.010$). Chironomid larvae were the dominant dietary item during the day (Fig. 5A), whereas notonectids (mainly nymphs) were dominant at night (Fig. 5B). The dominance of terrestrial insects increased from afternoon to

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Table 3. Results of generalized additive models with continuous predictor in interaction with site: A, time of the day; B, light intensity; and C, results of two separate models of surface and bottom oxygen concentration at site 3

A, time of the day				
Parametric terms		d.f.	*F*	*P*-value
Site		2	4.397	**0.0244**
Approximate significance of smooth terms	e.d.f.	Ref.d.f.		
Time:Site 1	1	1	0.727	0.403
Time:Site 2	4.881	5.96	11.825	**< 0.001**
Time:Site 3	4.581	5.616	10.483	**< 0.001**
B, light intensity				
Parametric terms		d.f.	*F*	*P*-value
Site		2	0.43	0.655
Approximate significance of smooth terms	e.d.f.	Ref.d.f.		
Light:Site 1	1	1	0	0.983
Light:Site 2	2.256	2.674	29.31	**< 0.001**
Light:Site 3	5.753	5.946	12.94	**< 0.001**
C, oxygen concentration				
Predictor	e.d.f.	Ref.d.f.	*F*	*P*-value
Bottom oxygen concentration	1.798	2.191	4.082	0.516
Surface oxygen concentration	1.934	2.404	4.713	**0.037**

Statistically significant predictors are indicated in bold.
Abbreviations: e.d.f., effective degrees of freedom; Ref.d.f., reference degrees of freedom.

midnight (Fig. 5C), and the dominance of Odonata larvae showed no temporal pattern (Fig. 5D).

DISCUSSION

Diel changes in activity are shaped by ecological and evolutionary constraints (Fry, 1947; Daan, 1981). In accordance with our predictions, we demonstrated that *Nothobranchius* annual killifish were generally active during daytime, with a single peak of activity around noon, although at the site with an exceptionally high population density (Vrtílek *et al.*, 2018a) no diel changes in activity were detected. Time, light intensity, surface temperature and oxygen concentration near the surface were all significantly associated with activity levels. Males were more active than females of all species. Unexpectedly, analysis of the spatial distribution of marked *N. furzeri* suggests that individuals move across the whole pool and lack permanent territories. Females ovulated their eggs only after sunrise, and most eggs were oviposited between late morning and mid-afternoon, with high feeding activity before oviposition of all oocytes. Chironomid larvae were the dominant diet during the day, whereas notonectids dominated at night.

Diel variation in locomotor activity is shaped by various requirements (such as feeding, social and reproductive activities) and constraints (such as environmental conditions or predator avoidance) (Noakes, 1992; Fraser *et al.*, 2004; Claireaux & Lefrançois, 2007). African annual killifishes maintained strong diurnal activity at two out of three sites (Fig. 1), consistent with variation in their activity in laboratory conditions (Lucas-Sánchez *et al.*, 2011; Almaida-Pagán *et al.*, 2018). A strong peak in activity at midday, during the period of highest light intensity, corresponded to the period of the day when visibility in the habitat was most favourable (Supporting Information, Fig. S2). We suppose that high visibility is important for effective foraging and for mate searching in these visually oriented micropredators. We speculate that a unimodal activity pattern in killifish is not suppressed by their potentially increased detectability from visually orienting avian predators (Haas, 1976b; Reichard *et al.*, 2018). In addition to light levels, water temperature and oxygen concentration were also strongly associated with the diel change in killifish locomotor activity, but they peaked and dropped later than activity and light intensity. This light–activity association is in accordance with observations from stable temperature and oxygen conditions in captivity, where light manipulation (turned on/off) has a clear impact on killifish activity (Lucas-Sánchez *et al.*, 2011; Almaida-Pagán *et al.*, 2018). In addition, oxygen concentration acts as a limiting factor on activity (Fry, 1947; McKenzie & Claireaux, 2010), especially when fish experience oxygen concentrations as low as 0.25 mg L^{-1}, as observed in our study (Supporting Information, Fig.

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S3). These low oxygen concentrations are characteristic for African (J. Žák and M. Vrtílek, personal observation) and South American ephemeral pools (Podrabsky *et al.*, 1998; Podrabsky & Wilson, 2016). Hence, low oxygen concentration can have an additional effect on diel activity changes in African killifish.

We acknowledge that the propensity to enter a trap is not necessarily associated only with locomotor activity but can be related to variation in personality traits (Biro & Dingemanse, 2009). For example, traps might be used by killifish as a shelter during the period of highest visibility to visually oriented predators. However, this explanation is at odds with the assumption of decreased antipredator response of African killifish in unpredictable environments (Polačik & Janáč, 2017). In addition, the increase in female bias over the season (Reichard *et al.*, 2014) does not indicate substantial antipredator behaviour among males. Across several species, traps were more frequently visited by bolder individuals (Wilson *et al.*, 1993; Biro & Dingemanse, 2009), which does not suggest that traps were used as shelters. Hence, we conclude that the entry of killifish into traps is most probably related to their activity or, potentially, to greater boldness in males. We accept, however, that the proximate cause of entrance into traps can only be disentangled in a dedicated laboratory experiment.

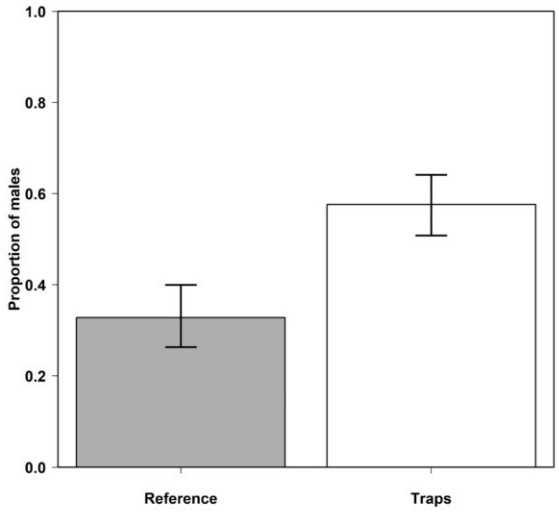


Figure 2. Males are the more active sex. The proportion of males in reference samples and trapping indicates their overrepresentation in traps. Data were pooled across species and sites (for sites 1–3). Whiskers represent 95% confidence intervals.

Fish activity is a plastic trait and responds flexibly to environmental cues (Reebs, 2002; Claireaux & Lefrançois, 2007) and physiological state (Metcalfe & Steele, 2001) despite strong constraints and trade-offs for the best timing of particular activities. The absence of any change in *Nothobranchius* activity at site 1 is likely to be related to the unusually high fish density [44 fish per standardized haul; compared with six to 13 fish per standardized haul from pools of comparable age (Vrtílek *et al.*, 2018a)], perhaps leading to physical interference between conspecifics, resulting in increased activity at night (Fingerle *et al.*, 2016). High fish density also reduces resources such as food and, consequently, slows growth (Post *et al.*, 1999). Indeed, fish at site 1 were smaller than usual for their age (Vrtílek *et al.*, 2019). Hungry fish are more active as they search for food (Metcalfe & Steele, 2001), and this may also have contributed to the observed modification of diel activity. Another possibility is that site 1 was strongly dominated by *N. orthonotus*, which might indicate that this species is not strictly diurnal. However, we do not believe this to be the case, because all 18 trapped *N. orthonotus* individuals at sites with normal fish density (sites 2 and 3; compare values in Table 1 with those of Vrtílek *et al.* 2018a) were captured during the day. In addition to mechanistic explanations (interference and persistent

Table 4. Section-specific analyses of *Nothobranchius furzeri* within-pool dispersal

Variable	χ^2	*P*-value	Prob > 0.05	χ^2	*P*-value	Prob > 0.05
	Section 1			Section 2		
Capture	6.17–6.69	0.214–0.245	0.685	3.24–3.53	0.415–0.447	0.949
Sex	6.01–6.52	0.218–0.248	0.707	4.97–5.32	0.246–0.274	0.836
Body size	7.45–7.95	0.132–0.155	0.554	2.74–2.96	0.475–0.508	0.979
	Section 3			Section 4		
Capture	2.35–2.61	0.547–0.581	0.968	1.60–1.75	0.660–0.688	0.996
Sex	2.31–2.53	0.542–0.575	0.985	3.74–4.09	0.385–0.424	0.902
Body size	2.55–2.79	0.506–0.539	0.980	7.72–8.28	0.143–0.169	0.515

Results are based on 1000 replicates of multinomial regression comparing the proportions of specific colour marks in the sample of recaptured fish with simulated datasets from repeated random sampling of all marked individuals. The 95% confidence intervals of χ^2 values and *P*-values from 1000 simulated tests are presented. The probability of the *P*-value from 1000 simulated tests being > 0.05 (Prob > 0.05) provides the likelihood of the *P*-value becoming non-significant. All tests are with three degrees of freedom.

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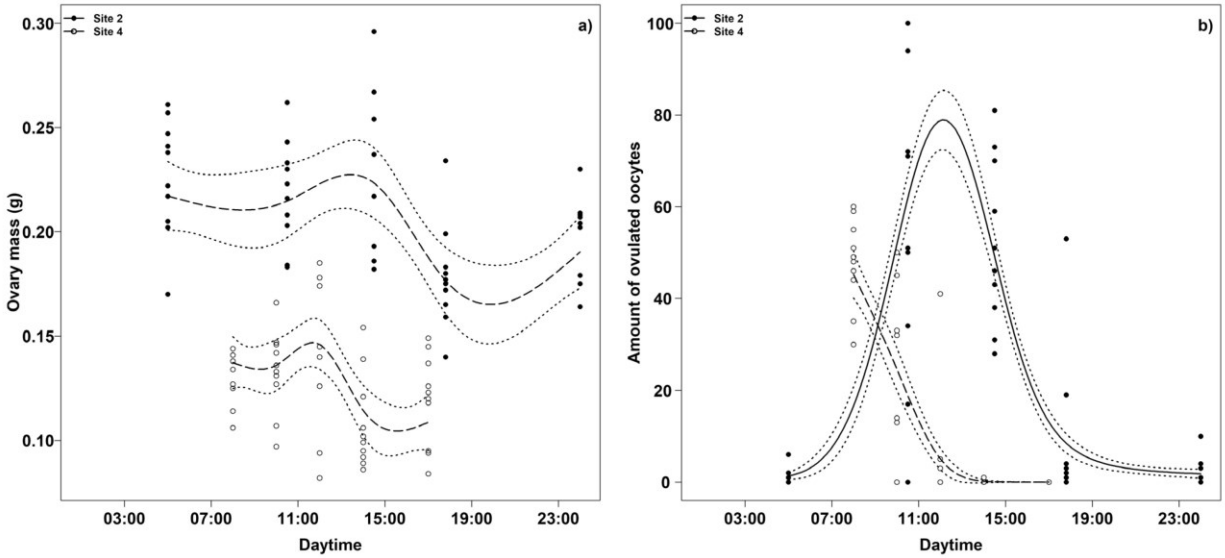


Figure 3. Reproductive parameters in *Nothobranchius furzeri* females. Generalized additive model (GAM) estimates from sites 2 and 4 demonstrate site-specific estimates of ovarian mass (GAM with a Gaussian error; A) and site-specific estimates of the number of mature oocytes (GAM with a zero-inflated Poisson error; B). Filled circles and continuous line represent estimates for site 2; open circles and dashed line represent estimates for site 4. Dotted lines are 95% confidence intervals.

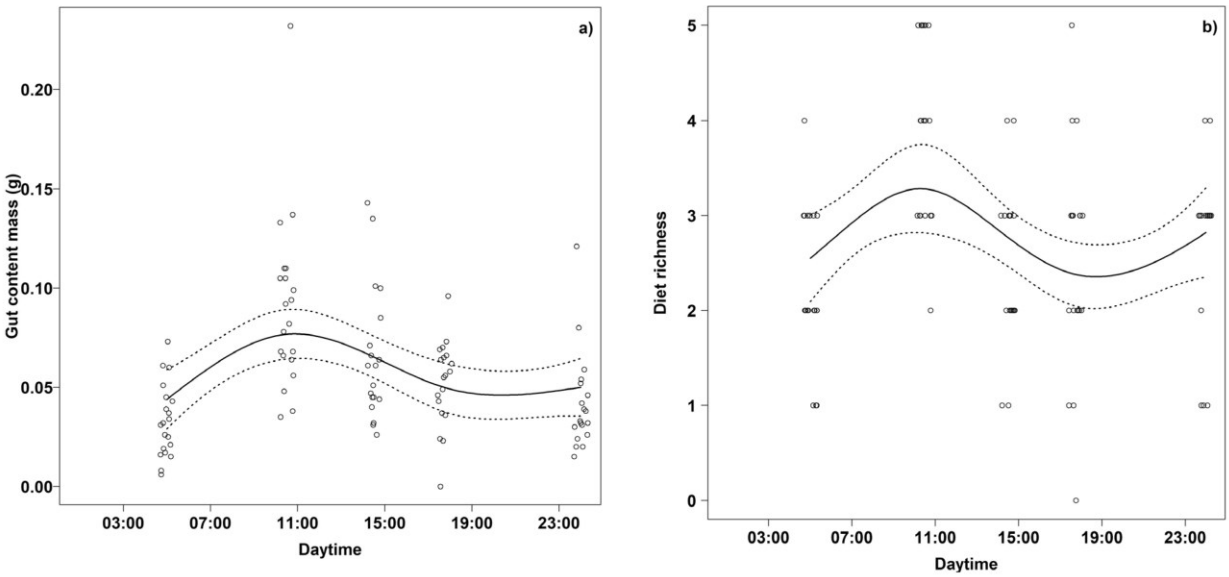


Figure 4. Diel dynamic of gut content mass and dietary richness in *Nothobranchius furzeri*. Model estimates of diel dynamic of gut content mass in both sexes of *N. furzeri* [generalized additive model (GAM) with a Gaussian error structure; A] and dietary richness (GAM with a quasi-Poisson error; B). Estimates of gut content mass are corrected for gut length. Estimates of dietary richness are corrected on gut content mass. Dashed lines represent 95% confidence intervals.

search for food), high population density and lack of food are major stressors, and activity patterns become aphasic in chronically stressed fish (MacFarlane & Livingston, 1983).

Sexual differences in locomotor activity are contingent on the mating system (Perrin & Mazalov, 2000; Croft *et al.*, 2003) and result in increased risk of sex-specific predation (Haas, 1976b). We found that males were

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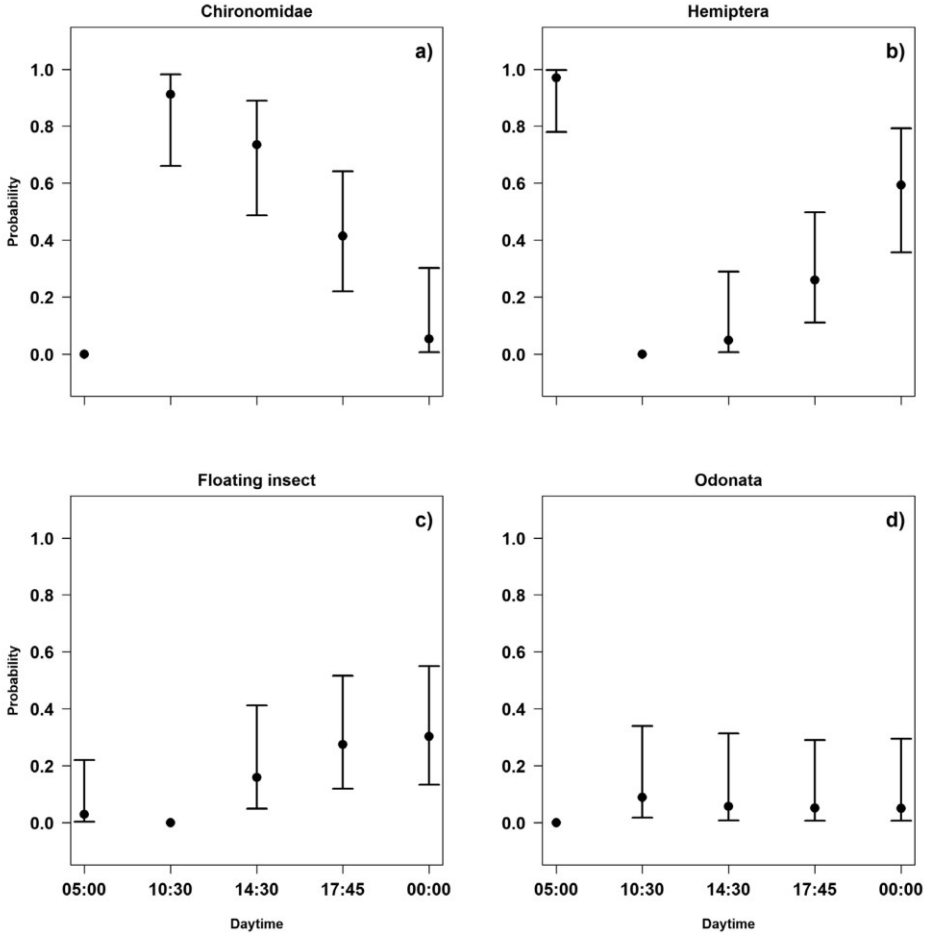


Figure 5. Diel change in the probability of dominance in the gut of *Nothobranchius furzeri* from site 2 in the four most frequently dominant dietary categories. Results are derived from multinomial regression, with time of day as a fixed factor. Error bars represent 95% confidence intervals.

more active than females across all three species. Spawning in African killifishes involves males actively pursuing females (Polačik & Reichard, 2011). High male activity conceivably increases their reproductive success, in a similar manner to Trinidadian guppies (*Poecilia reticulata*), in which active males enjoy higher mating success (Croft *et al.*, 2003). Higher male activity exposes them to a higher risk from ambush predators, such as giant *Belostoma* bugs (Heteroptera) and piscivorous birds (Haas, 1976b; Reichard *et al.*, 2014), thus female-biased natural populations are common in the wild (including the sites examined in this study) despite equal sex ratios at maturation (Reichard *et al.*, 2014; Vrtílek *et al.*, 2018a).

Male territories in the form of spawning arenas have been suggested in wild *N. furzeri* (Reichard *et al.*, 2009). Our data on dispersal of *N. furzeri* within the entire pool over a 24 h period contradict the suggestion of permanent territories. Marked fish dispersed across the entire pool over a short period, with no difference between males and females. It is possible that *Nothobranchius* males possess short-term spawning arenas that are dissolved at night and resettled the next day (Lucas *et al.*, 2001), with no individual connection to the arena occupied the previous day. We speculate that the mating behaviour of *Nothobranchius* males involves active pursuit of females and opportunistic use of spawning sites rather than signalling and oviposition in permanent territories. Nevertheless, the possibility that random redistribution of *N. furzeri* is a consequence of disturbance caused by our capture, release and recapture efforts cannot be excluded entirely. Although our data suggest that the African killifish mating strategy does not include permanent territories or spawning arenas, we acknowledge that only direct observation of male behaviour in natural conditions might ultimately resolve the potential existence of

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short-term male-defended locations that are observed in the laboratory, where the oviposition substrate is spatially constrained (Polačik *et al.*, 2016).

One of the behavioural conflicts is between individual maintenance and reproduction (Cuthill & Houston, 1997; Heino & Kaitala, 1999). Activities directly associated with individual maintenance, such as feeding, are usually prioritized over reproduction, and when nutritional requirements are not met, reproduction can cease (Tyler & Dunn, 1976). Such behavioural conflict is especially critical in short-lived income breeders, such as annual killifish. The highest scores of gut fullness and dietary richness in morning samples demonstrate that feeding is prioritized over reproduction early in the morning in *N. furzeri*. This is similar to the temporal partition of those behaviours (morning peak in feeding activity, afternoon peak in reproduction) in the Amargosa pupfish (Lema *et al.*, 2010).

Annual killifish are income breeders and expend energy on reproduction on a daily basis (Blažek *et al.*, 2013; Vrtílek & Reichard, 2015), resulting in diel periodicity of gonad status (Vrtílek & Reichard, 2016). We recorded the highest ovarian mass in the morning, with a decline from noon to evening. This decrease implies an average of 4% of eviscerated body mass (estimated as the average decrease in ovarian mass between morning and afternoon samples) spent daily on eggs production. Contrary to females, males displayed no diel change in gonad mass.

Light conditions appear to trigger ovulation in *Nothobranchius*. Several environmental cues (such as photoperiod or temperature) modulate egg ovulation (i.e. release of the mature egg from its follicle, making it ready for oviposition) in fishes (Wallace, 1978; Wang *et al.*, 2010). For example, the light–dark cycle governs oviposition in medaka (*Oryzias latipes*) (Ueda & Oishi, 1982; Weber & Spieler, 1987). It was believed that the eggs of *Nothobranchius* females are ovulated overnight (Vrtílek & Reichard, 2016) or shortly after sunrise (Haas, 1976a). We found that *Nothobranchius* females had almost no ovulated eggs at night, even as late as 1 h before sunrise (at 05.00 h), but ovaries were full of ovulated eggs shortly after sunrise (08.00 and 10.30 h). Although a sharp increase in light intensity over that period coincides with the changes in water temperature, laboratory studies report the same cyclicity of ovulation in constant temperature conditions (Blažek *et al.*, 2013; Vrtílek & Reichard, 2015). The duration of reproductive activity (i.e. the interval between the peak number of ovulated eggs in the ovaries and the time by which all eggs were spent) varied between populations. The different temporal estimates of peak egg abundance in ovaries, revealed in our analysis, could be related to slightly different sampling schedules between the two populations. Variation in the time by which all eggs were spent was likely to be related to population density and availability of males, because females spent their ovaries sooner at site 4, where males were more abundant (Table 1). We suggest that sunrise ovulation might be a common trait for the *Nothobranchius* genus in the wild, because this has also been observed in *Nothobranchius guentheri* in a mesocosm (Haas, 1976a).

The extremely condensed life history of annual killifish (Vrtílek *et al.*, 2018b) and daily reproduction require a high energetic income. This requires substantial feeding activity, with diel patterns driven by trade-offs such as reproduction and/or prey detectability, which is related to light intensity (Maglio & Rosen, 1969; Lema *et al.*, 2010). Our data suggest that feeding is incessant; the guts of *N. furzeri* were never empty (except for a single male), including samples collected at night (00.00 and 05.00 h). Slow digestion and excretion time is an unlikely alternative explanation, because the diet differed qualitatively over the diel cycle. During daytime, the diet was dominated by chironomid larvae, coupled with an increase in the proportion of terrestrial insects in late afternoon. At night, notonectids (mainly nymphs) dominated the diet. Different digestion time among different prey is an unlikely explanation of the diel shift in diet, because most identified food items, whether recovered during the day or the night, had at least some indigestible and easily detectable hard chitinous structures, such as head capsules in chironomids, notonectid body or fragments of terrestrial insects.

Cyclical changes in dominant prey have been observed in other small-bodied fishes, such as mosquitofish, *Gambusia affinis* (Maglio & Rosen, 1969), and dwarf panchax, *Aplocheilus parvus* (Fernando *et al.*, 2015), and ascribed to variation in habitat use over the diel period (Maglio & Rosen, 1969). Owing to the strong spatial constraints of annual killifish habitats, we suggest that temporal differences in diet are related to variation in prey accessibility and detectability rather than to shifts in habitat use. Hence, we speculate that chironomid larvae are easily detectable visually during the daytime but not at night; notonectids might be detected at night via the fishes' lateral line or by contrast against the sky, and we suggest that the peak in terrestrial insects in late afternoon corresponds to the activity patterns of these insects (Reynoldy & Riley, 1979; Kasumyan, 2003).

We note that a very low proportion of notonectids was previously reported in the diet of *Nothobranchius* (Polačik & Reichard, 2010; but see Wildekamp, 1983), in contrast to our present study. In addition to differential prey availability among populations, we argue that differences in sampling time largely contributed to that difference. Samples

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for the previous study on the diet of Mozambican *Nothobranchius* fishes (Polačik & Reichard, 2010) were collected at midday, before notonectids (and also terrestrial insects) started to form a substantial proportion of the diet.

In conclusion, we provide the first empirical description of diel activity in three wild populations of African killifish. All three *Nothobranchius* species studied are diurnal fishes with a single peak of locomotor activity around noon, with the exception of one densely populated site, where fish remained active throughout the 24 h cycle. Higher locomotor activity in males is likely to be associated with their active reproductive strategy, although their larger size might also intensify their activity. Individual *N. furzeri* move across the whole pool and do not occupy permanent territories. It appears that feeding is prioritized over reproduction in the morning, because gut fullness and dietary richness peak before all mature oocytes are spent. The composition of the diet varies over the diel cycle, primarily altering between dominance of chironomid larvae during the day and notonectid nymphs at night. Our results contribute to the understanding of the natural life history of African annual killifishes and provide baseline data for further laboratory studies of diel patterns in locomotor activity and their changes over the short *Nothobranchius* lifespan.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Surface and bottom water temperature (in degrees Celsius) over a 24 h period at sites 1–3 (A–C), measured during trapping. Black line shows temperature measured at pool surface (5 cm below water surface), and grey line represents bottom temperature (measured directly on the substrate). Logging interval was set to 1 min.
Figure S2. Light intensity (in lux) on water surface over a 24 h period at sites 1–3 (A–C) (measured during trapping). Logging interval was 1 min.
Figure S3. Oxygen concentration (in milligrams per litre) at site 3 over a 24 h period of fish trapping. Red line and points represent oxygen concentration measured at the pool surface (5 cm below the surface). Blue line and points represent values measured 10 cm above the pool substrate. Each point represents the mean value of three independent measurements performed at three different parts of the pool.
Table S1. Table of sample sizes used in analysis of activity, feeding and reproduction rhythmicity of the African annual killifish from genus *Nothobranchius*. Abbreviations: NF, *Nothobranchius furzeri*; NO, *Nothobranchius orthonotus*; NP, *Nothobranchius pienaari*.
Table S2. Quantitative comparison of candidate models (generalized additive models with Gaussian error) of fish activity at site 3 and their results. Degrees of freedom (d.f.) absolute Akaike information criterion (AIC), difference from the best model (ΔAIC) and their statistics, estimated degrees of freedom (e.d.f.), *F*-statistics and *P*-value, are shown for models, with time of day, light intensity, surface and bottom temperatures, surface and bottom oxygen concentrations as explanatory variables.

SHARED DATA

Original data are available at the Figshare repository (doi: 10.6084/m9.figshare.7326425).

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PAPER 8: Thermal preferendum

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Limited differentiation of fundamental thermal niches within the killifish assemblage from shallow temporary waters

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ABSTRACT

The coexistence of ectothermic species is enabled among other factors by the differentiation of their thermal niches. While this phenomenon is well described from deep temperate lakes, it is unclear whether the same pattern applies to temporary pools. In this study, we examined fundamental thermal niches in three coexisting annual killifish species *Nothobranchius furzeri*, *N. orthonotus* and *N. pienaari* from temporary pools in southern Mozambique. We hypothesized that the disparate thermal requirements of the three congeneric species are a candidate niche component that facilitates their local coexistence. We estimated species' thermal requirements as preferred body temperatures (T_{pref}) in a horizontal thermal gradient. Under thermal gradient conditions, sympatric killifish maintained their body temperatures within similar T_{pref} ranges despite some variation in mean T_{pref}. The daily variation in water temperature in their native habitats enables killifish to thermoregulate at least for part of the diurnal cycle. We conclude that the coexistence of African annual killifish species is possible without the differentiation of their fundamental thermal niches.

1. Introduction

Environmental temperature is one of major factors determining species occurrence in a given habitat. Although environmental temperature represents a stress factor rather than a resource (Chase and Liebold, 2003), thermal requirements constitute an important part of a species' ecological niche because of the pervasive effect of temperature on organismal functions and processes (Magnuson et al., 1979). Implicit in the recent definition of ecological niche (Chase, 2011), thermal niche is the range of body temperatures at which positive population growth occurs (Bennett and Lenski, 1993; Gvoždík, 2018). This pertains mainly to ectotherms, because their body temperatures are directly influenced by environmental (operative) temperatures. To maximize population growth, ectotherm target body temperatures should be readily attainable in a given habitat. However, species occupying the same area often vary in their thermal niches (Magnuson et al., 1979). Although knowledge of organisms' thermal niches provides invaluable information for fundamental ecological issues, such as the impact of climate change on ectotherm populations (Dulvy et al., 2008) and the potential coexistence of ecologically similar species (Ding et al., 2018; Ohlberger et al., 2008) thermal niche variation within ecological communities is not fully understood.

Several scenarios may explain thermal niche differentiation ranging from interspecific competition for shared limited resource(s), through past competitive exclusion of species with similar requirements and divergent selection to the recent occupation of the same area by species with disparate thermal requirements (Paterson and Blouin-Demers, 2017). Thermal niche differentiation is well known from various terrestrial and aquatic communities (Ding et al., 2018; Magnuson, 2010; Mushinsky et al., 1980). Deep temperate lakes are particularly illustrative of this phenomenon, because fish species are separated across the water column according to their thermal requirements (Larsson, 2005; Magnuson, 2010; Ohlberger et al., 2008). In contrast to lakes, information about thermal niche differentiation in shallow water bodies is scarce.

Shallow water bodies provide a thermally challenging environment to ectotherms due to both high daily temperature fluctuations (Heath et al., 1993; Reichard et al., 2009) and the occurrence of extreme temperatures, i.e. above 40 °C (Heath et al., 1993; Podrabsky et al., 1998; Reichard, 2016). The temperature range may reach 15–20 °C between midday and early morning (Podrabsky et al., 2008; Reichard et al., 2009). A depth of as little as 15 cm contains sufficient variation for daytime thermal stratification (Hadamová and Gvoždík, 2011). The difference between bottom and surface temperature may be as much as 15 °C, providing the opportunity for behavioral thermoregulation in aquatic organisms (Hadamová and Gvoždík, 2011). In comparison to

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large water bodies, the opportunity for behavioral thermoregulation in temporary pools is more time-limited, potentially increasing interspecific competition for space at suitable water temperatures. Accordingly, this might lead to thermal niche differentiation as commonly observed in lakes (see references above).

In this study, we examined the fundamental thermal niches of three coexisting species of *Nothobranchius* spp. from southern Mozambique. Fundamental thermal niche was estimated using the optimality paradigm (MacArthur and Pianka, 1966; Schoener, 1971; Stephens and Krebs, 1971). This assumes that under the absence of any biotic and abiotic limitations, ectotherms are able to maintain body temperatures that maximize population growth, i.e. preferred body temperatures. The three species, *N. furzeri, N. orthonotus* and *N. pienaari* are closely related (Dorn et al., 2014) and their distributions strongly overlap (Reichard et al., 2017b). They commonly occur in a single pool with subtle microhabitat preferences and high trophic niche overlap (Polačik et al., 2014; Polačik and Reichard, 2010; Reichard et al., 2017b, 2009). Accordingly, we hypothesized that disparate thermal requirements among the three congeneric species are a candidate niche component that facilitates their local coexistence. To test this hypothesis, we compared T_{pref} ranges not only among species, but also with temperatures that fish potentially experience in their native habitats, i.e. the opportunity for behavioral thermoregulation (Hadamová and Gvoždík, 2011).

2. Materials and methods

2.1. Study species and maintenance

Nothobranchius furzeri, N. orthonotus and *N. pienaari* are small (4–8 cm) cyprinodontiform fishes inhabiting ephemeral savanna pools in southern Mozambique. They are primarily differentiated by male nuptial coloration (Wildekamp, 2004), with ecological differentiation from a generalized form restricted to a limited number of species (Costa, 2018). The separation of the three species was estimated to be 3–4 mya (Dorn et al., 2014), which is probably sufficient time for any thermal niche divergence to occur.

We used populations of *N. furzeri* (population MZCS 002 after Bartáková et al., 2015), *N. orthonotus* (MZCS 002) and *N. pienaari* (MZCS 505) from the center of the area where all three species are sympatric. All study populations originated from the same region (the northern bank of the River Limpopo, Gaza province, Mozambique). Experimental individuals were 3rd to 4th generation descendants of wild-caught fish that were bred in large captive populations to minimize inbreeding (Blažek et al., 2016). All generations were kept at the breeding facility of the Institute of Vertebrate Biology in Brno, Czech Republic.

Experimental fish were hatched on 1 August 2016 according to the standard protocol for *Nothobranchius* species (Polačik et al., 2016). Fish were housed in 60 L aquaria with approximately 30 individuals of both sexes. Some males of the most aggressive species, *N. orthonotus,* were kept separately from females to prevent male-male aggression, female harassment, and potential injuries. Fish were fed once per day ad libitum with frozen bloodworms (chironomid larvae). The light regime was set to 14 L:10D. Aquarium water temperature was maintained at 26 ± 1 °C. This water temperature was chosen because it represents the typical mean ambient temperature in natural pools. Water (one third of total volume) was changed twice per week.

For the purpose of this study, we used a total of 30 adult individuals per species (Table 1). Once they reached the age of 11 weeks, fish were transported in groups of 12–15 (a combination of all species and sexes) to the experimental facility of the Institute of Vertebrate Biology in Studenec, Czech Republic. In the experimental facility, three to five individuals of the same species were kept in 20 L tanks. The thermal, light, and feeding regimes were identical to those in the breeding facility. Fish were kept under these conditions for at least two days before measurements. All experimental data were collected between 20 October and 19 November 2016.

Table 1
Sample size and body mass (mean (SD)) of three *Notobranchius* species used in this study.

Species	Sample size		Body mass (g)	
	Females	Males	Females	Males
N. furzeri	15	15	0.83 (0.13)	1.74 (0.85)
N. orthonotus	19	11	0.94 (0.30)	1.15 (0.45)
N. pienaari	15	15	0.31 (0.05)	0.66 (0.11)

2.2. Preferred body temperatures

We measured preferred body temperature (T_{pref}) in a horizontal thermal gradient (15.5–38.5 °C) maintained in a stainless steel tank (240 × 60 × 60 cm). The tank was divided into three lanes. Each lane had 12 partially separated compartments (20 × 20 cm) that varied in water temperature. Partitions were made of 0.5 cm styrofoam with a 3 × 3 cm opening to enable fish movement between compartments. The water temperature increased by 2.0 ± 0.5 °C with each compartment along the horizontal gradient. Water temperature was maintained by the combination of Peltier modules mounted under the bottom of each compartment together with an aquatic heat recuperation system connected to a programmable control unit (see Gvoždík, 2003 for further details). Each compartment was gently aerated to prevent water temperature stratification. Temperatures were measured using calibrated thermistor probes connected to dataloggers (resolution 0.01 °C; UX120-006M, HOBO, Onset Computer, Bourne, MA, USA) three hours before the start of recording and again no longer than one hour after terminating a trial. The mean temperature calculated from both values was used as the representative water temperature for each compartment. After a trial, the tank was re-filled with non-chlorinated well water to the depth of 3 cm. The room temperature was kept at 26 ± 1 °C to maintain the stability of the thermal gradient during trials.

We placed three randomly chosen fishes separately into one lane of the thermal gradient for 15 h before the start of trial. Each fish was placed into the compartment with the same water temperature as in the stock aquarium. Fish motor activity was recorded during the following day, between 10:00 and 17:00, using a digital camera surveillance system (12 fps; MTV-63S80H-A-ICR-R, Mintron, Taipei, Taiwan). This time window matches the period when natural pools become thermally stratified and fish have an opportunity to thermoregulate (Fig. 1). Fishes were not fed during the experimental trial, because a pilot study confirmed that feeding had a slight influence on T_{pref} in each species. After the trial, each fish was weighed to the nearest 0.01 g (Kern EG, Kern, Balingen, Germany). All fishes were used only once in the experiment and returned back to the breeding facility for stock maintenance after measurement.

From the videos, we recorded the horizontal positions of fishes in the gradient at 10 min intervals. Average T_{pref} over six intervals each hour was used for statistical analysis. The body temperature of small fish rapidly converges with that of the surrounding water (Lutterschmidt and Hutchison, 1997), and thus we estimated fish body temperatures by extrapolating from water temperature at given horizontal positions. The lower (LBT_{pref}) and upper (UBT_{pref}) boundaries of preferred body temperature were calculated from individual T_{pref} distribution as the 10th and 90th percentile, respectively. We assume that these values delimit the target range of T_{pref} more accurately than the narrower or parametric estimates (Hertz et al., 1993; Magnuson et al., 1979). In addition, thermal dependence of the key physiological process, aerobic scope, supports a wider rather than narrower estimate of T_{pref} range (Gvoždík and Kristín, 2017). The range of preferred body temperatures was calculated as the difference between UBT_{pref} and

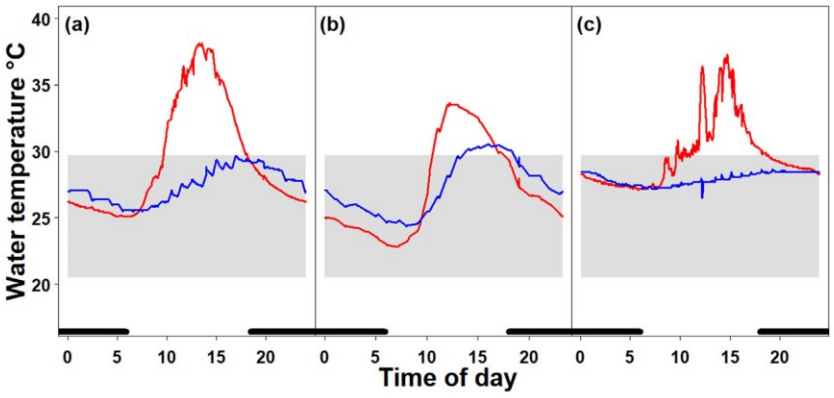


Fig. 1. Diel variation in surface and bottom water temperatures in three pools with *Nothobranchius* spp. in Mozambique. The grey area represents the range of preferred body temperatures averaged across three species, *N. furzeri, N. orthonotus,* and *N. pienaari,* given only minor interspecific variation in this trait (see Fig. 2). Horizontal black bars denote the night period. (a) Maximum depth 35 cm, (b) maximum depth 20 cm with canopy cover, (c) maximum depth 95 cm.

LBT_{pref} (i.e. central 80% of all measurements). For comparative purposes, we repeated our analyses with T_{pref} boundaries calculated as 25th and 75th percentiles.

The motor activity of each fish was recorded over seven 10 min intervals during trial and the number of times a fish crossed between compartments was summed for activity analysis. Four individuals (*N. furzeri* two females and one male; *N. pienaari* one male), which swam continuously across thermal gradients during the whole trial, and one inactive individual (*N. pienaari* male) were discarded from further analyses.

2.3. Field water temperatures

To obtain information about the daily temperature range that fish experience in the wild, we recorded water temperatures at three water bodies (Fig. 1) with the co-occurrence of at least two killifish species using temperature data loggers (two per site; HOBO, UA-002–64, Onset Computer, Bourne, MA, USA). The first datalogger was placed at the maximum depth on the bottom. The second datalogger was anchored 5 cm under water surface and shaded from direct sunlight. Temperatures were recorded at 1 min intervals over a 24 h period. Using these values and fish T_{pref} ranges, we calculated the opportunity for thermoregulation, which is the proportion of daytime that allows thermoregulation, i.e. maintaining body temperatures within the T_{pref} range, if the range of vertical thermal gradient > T_{pref} range (Hadamová and Gvoždík, 2011).

2.4. Statistical analyses

We applied a linear mixed model to analyze the effect of species identity on T_{pref}. The full model contained three fixed effects: species, sex, and experimental lane. Body mass, activity and time of day were added as covariates. The interactions between factors were also included. Random factors included both "individual identity" (intercept) and "time" (slope). We used a model simplification approach; the minimal adequate model was selected according to the lowest Akaike Information Criterion corrected for small sample size (AICc).

The ranges of T_{pref}, UBT_{pref} and LBT_{pref} were analyzed using a general linear model. The full model contained five factors: species, sex, body mass, gradient lane, mean T_{pref}, and species × sex interaction. The most parsimonious model with the lowest AICc was chosen by backward selection. In all models, the distribution of residuals was checked via diagnostic plots. Tukey *post hoc* tests were used for all interspecific comparisons. Fish motor activity was analyzed with a generalized linear model with a quasi-Poisson distribution because of high overdispersion. The full model contained three factors: species, sex, and gradient lane, and their interactions. Body mass was added as a covariate. All statistical analyses were performed in R using the "nlme" (Pinheiro et al., 2016), "MuMIn" (Bartoń, 2016) and "lsmeans" (Lenth, 2016) packages.

3. Results

In total, we obtained 591 T_{pref} measurements from 85 individuals of three species (five individuals were excluded, see above). The species varied in their mean T_{pref} ($F_{2,82}$ =10.52, $P < 0.001$; Fig. 2). Specifically, *N. orthonotus* maintained higher mean T_{pref} than both *N. furzeri* (Tukey HSD test, $P = 0.03$) and *N. pienaari* ($P < 0.001$), while the latter two were similar ($P = 0.08$). Generally, individual mean T_{pref} decreased with locomotor activity level ($F_{1,498} = 45.74$, $P < 0.001$). Individual identity (random intercept) and individual variation in the time course of body temperatures (random slope) explained 88% of total variation in T_{pref}.

The T_{pref} range was similar across the species (Fig. 2). Active individuals had a broader T_{pref} range than less active individuals ($F_{1,79} = 8.33$, $P = 0.005$). At the individual level, LBT_{pref} and UBT_{pref} were positively related to mean T_{pref} (LBT_{pref}: $F_{2,81} = 137.55$, $P < 0.001$; UBT_{pref}: $F_{2,81} = 132.07$, $P < 0.001$). Therefore, we used mean T_{pref} as a covariate to analyze the influence of species identity on these traits. The T_{pref} range boundaries were consistent among species (LBT_{pref}: $F_{2,81} = 1.78$, $P = 0.17$; UBT_{pref}: $F_{2,81} = 1.88$, $P = 0.16$; Fig. 2). Qualitatively identical results were obtained for T_{pref} boundaries calculated as 25% and 75% percentiles (Table S1, Fig. S1).

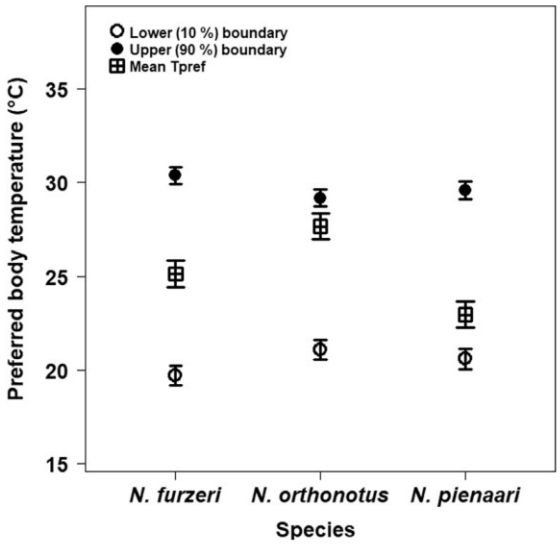


Fig. 2. Mean preferred body temperature, lower, and upper boundaries of preferred body temperature range in three *Nothobranchius* species. Boundaries were calculated as 10th and 90th percentile of individual body temperature distribution. Means (± SE) are model-estimated parameters.

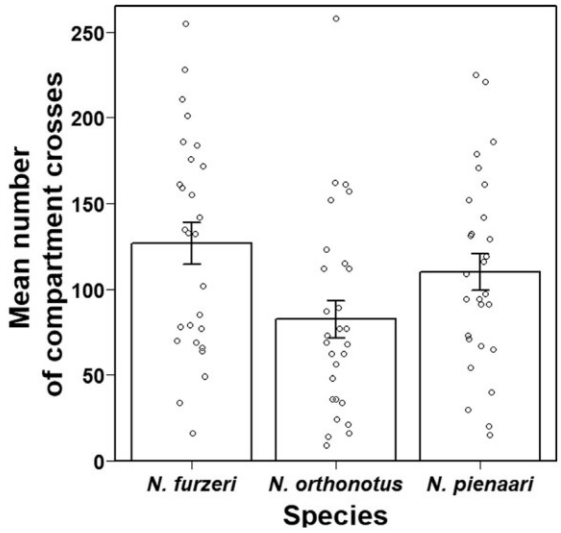


Fig. 3. The motor activity of three *Nothobranchius* species in a thermal gradient. Activity was estimated as the sum of compartment crosses over seven 10 min intervals. Data points were jittered horizontally to improve their visibility. Error bars represent 1 SE.

The index of locomotor activity varied among species ($\chi^2 = 7.82$, $P = 0.02$; Fig. 3). Specifically, *N. furzeri* was more active than *N. orthonotus* ($z = 2.74$, $P = 0.02$). The locomotor activity of *N. pienaari* did not differ from the other two species (NP vs. NF: $z = 0.99$, $P = 0.59$; NP vs. NO: $z = 1.79$, $P = 0.17$).

The daily temperature range in all temporary pools enabled killifish to thermoregulate for at least part of the day. In two deeper pools, killifish could behaviourally thermoregulate for 7–8 h per day (31–32% of the diurnal cycle; Fig. 1a, c). In the shallowest pool, thermoregulation was possible for 2.5 h only (10% of the diurnal cycle; Fig. 1b).

4. Discussion

Thermal niche often varies among species occupying the same area (Magnuson et al., 1979; Ohlberger et al., 2008). In contrast to these findings, our results show that the coexistence of African annual killifish species is possible without the differentiation of their fundamental thermal niches. In the laboratory thermal gradient, the killifish species maintained their body temperatures within the same ranges, though their mean values varied. We found a high level of variation in T_{pref} among individuals, which was related to their locomotor activity within the gradient. Below, we discuss these findings within the context of annual killifish ecology and the methodology of T_{pref} measurements.

Similarities in fundamental thermal niches suggest that (i) the killifish species were equally adapted to thermal conditions in temporary pools or (ii) their thermal niches were inherited from a common ancestor 3–4 mya (Dorn et al., 2014). On the one hand, the latter possibility seems surprising, because annual killifishes are a fast evolving species (Reichwald et al., 2015; Valenzano et al., 2015), and this time period should be sufficient for thermal niche differentiation. On the other hand, T_{pref} is often conserved within genera (Angilletta and Werner, 1998; Youssef et al., 2008; but see Labra et al., 2009). Specifically, the evolutionary rate of the upper T_{pref} boundary is relatively slow (Gvoždík, 2015), which may prevent thermal niche differentiation by increasing UBT_{pref}, even when diel thermal profiles in their native habitat provide the potential for it (Fig. 1). The limited thermal niche differentiation among annual killifish seems even weaker than trophic niche partition (Polačik et al., 2014; Polačik and Reichard, 2010), which concurs with the general ecological similarity of these species (Wildekamp, 2004). Hence, further studies will investigate whether the co-occurrence of these taxa is determined by their differentiation in other niche dimensions, ecological trade-offs (Kneitel and Chase, 2004), or negative frequency dependence (Svensson et al., 2018).

Alternatively, because our fish were kept in stable temperature for 3–4 generations after import from the wild, we cannot rule out that this approach reduced intra- and interspecific variation in T_{pref} due to a rapid evolutionary response to the captive environment (Christie et al., 2012; Araki et al., 2007). However, as we mentioned above, the T_{pref} range seems a phylogenetically conservative trait (Gvoždík, 2015). Hence, we assume that our results were unbiased by captive rearing. Untangling the interactive effect of genetic, developmental, and environmental factors on T_{pref} variation in this system provides an interesting research agenda for further studies.

The T_{pref} range of the species we examined was almost identical to that previously observed in a South American annual killifish, *Austrofundulus limnaeus,* inhabiting thermally similar habitats (Podrabsky et al., 2008). A likely factor contributing to the wide T_{pref} ranges in these taxa is unpredictable thermal variation, and accordingly the opportunity for thermoregulation (Hadamová and Gvoždík, 2011), in their native habitat both within and among generations (Gilchrist, 1995; Reichard et al., 2017a). Under these conditions, the wide T_{pref} range may reduce the time and energy spent in behavioral thermoregulation (costs of thermoregulation), which can be invested in other activities such as feeding or reproduction (Angilletta, 2009; Huey and Slatkin, 1976).

Despite negligible variation in T_{pref} range, mean T_{pref} varied among species. Although all three species moved across the same temperatures in the thermal gradient, *N. orthonotus* spent more time at warmer temperatures than *N. furzeri* and *N. pienaari*. The cause of this variation is unknown. Perhaps because the T_{pref} range provides the best thermal compromise for various biochemical processes and physiological functions within an organism (Cossins and Bowler, 1987; Gvoždík and Kristín, 2017; Hochachka and Somero, 2002), variation in mean T_{pref} may reflect interspecific variation in acute demands to maximize performance of some physiological traits over others. In addition, the high individual variation in T_{pref} and the non-random distribution of "low-"and "high-T_{pref}" individuals among species may also contribute to this result. Anyway, this finding clearly demonstrates that characterizing T_{pref} using only its mean value may produce misleading information about the magnitude of intra- and interspecific variation in this trait.

During T_{pref} trials, locomotor activity level varied among killifish species. Individuals that maintained higher body temperatures increased their activity relative to individuals with lower T_{pref}. A similar relationship between activity and T_{pref} has been reported in other fish species, *Danio rerio* and *Oreochromis niloticus* (Cerqueira et al., 2016; Rey et al., 2015). This pattern may be related to "thermal-behavioral syndrome" (Michelangeli et al., 2017), which predicts that the T_{pref} of each individual fits somewhere within the cold–hot continuum and the position on this continuum directly influences individual personality, e.g. activity level, boldness, and accordingly habitat utilization. High (88%) individual variation in T_{pref} also concurs with this prediction. This individual variation in T_{pref} may be beneficial for reducing intraspecific competition in the wild (Beitinger and Magnuson, 1975; Medvick et al., 1981). Given the similar thermal requirements among taxa, individual T_{pref} variation may reduce not only intra- but also interspecific competition within the African annual killifish assemblage.

In conclusion, we showed that syntopic coexistence of three annual killifish is possible without differentiation in their fundamental thermal niche. Their wide T_{pref} ranges seem well suited to life in thermally heterogeneous temporary pools in the African savanna. However, a wider comparative study including more taxa distributed across a latitudinal gradient will help to resolve whether similar thermal requirements result from evolutionary conservation of this trait (Gvoždík, 2015; Muñoz et al., 2014) or adaptation to thermal conditions in their common habitat (Labra et al., 2009). Next, we demonstrated that focusing solely on mean T_{pref} may produce misleading results about the

trait variation. Preferred body temperature has remained the most meaningful measure in thermal ecology for decades (Huey, 1982). However, their accurate estimates require not only controlling for many confounding factors (Hutchison and Dupré, 1992), but also choosing suitable parameters characterizing this trait. As has been shown before (Gvoždík, 2015), the T_{pref} range is much more informative than mean T_{pref}, especially from the perspective of thermal niches (Gvoždík, 2018). The most comprehensive approach to estimating T_{pref} should also include information about ectotherm locomotor activity in the thermal gradient, because it may be associated with T_{pref} parameters. Finally, though mean T_{pref}s concur with previously published general recommendations on *Nothobranchius* spp. husbandry (Dodzian et al., 2018; Genade, 2005; Polačik et al., 2016), our study provides the first information about the thermal biology of these species, which are currently used as models in aging research (Cellerino et al., 2016; Hu and Brunet, 2018).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtherbio.2018.10.015.

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PAPER 9: Senescence under fluctuating temperature

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Fluctuating temperatures extend median lifespan, improve reproduction and reduce growth in turquoise killifish

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ABSTRACT

In natural populations, individuals experience daily fluctuations in environmental conditions that synchronise endogenous biorhythms. Artificial alterations of environmental fluctuations can have negative consequences for life history traits, including lifespan. In laboratory studies of aging, the role of fluctuating temperature is usually overlooked and we know little of how thermal fluctuation modulates senescence in vertebrates. In this longitudinal study we followed individually-housed turquoise killifish, *Nothobranchius furzeri*, from two thermal regimes; ecologically relevant diel fluctuations (20 °C - 35 °C) and stable temperature (27.5 °C), and compared their survival, growth and reproduction. Fish experiencing fluctuating temperatures had a longer median lifespan but reached smaller asymptotic body size. Within-treatment variation indicated that extended lifespan in fluctuating temperatures was not causally linked to decreased growth rate or smaller body size, but occurred solely due to the effect of thermal fluctuations. Male body size was positively associated with lifespan in stable temperatures but this relationship was disrupted in fluctuating thermal regimes. Females exposed to fluctuating temperatures effectively compensated egg production for their smaller size. Thus, there was no difference in absolute fecundity between thermal regimes and body-size corrected fecundity was higher in females in fluctuating temperatures. Overall, despite a brief exposure to sub-optimal thermal conditions during fluctuations, fluctuating temperature had a positive effect on survival and reproduction. These results suggest that the expression of life history traits and their associations under stable temperatures are a poor representation of the relationships obtained from ecologically relevant thermal fluctuations.

1. Introduction

Environmental conditions modulate most organismal functions, from gene expression to behaviour (Fraser et al., 1993; Podrabsky and Somero, 2004). Temperature, light intensity and food availability fluctuate in predictable daily and seasonal cycles and organisms adapt their endogenous biorhythms to optimize performance (Claireaux and Lefrançois, 2007; Dillon et al., 2016). On the other hand, environmental fluctuation exposes organisms to periods of suboptimal conditions which may have negative consequences for survival (Zhang et al., 2018). Artificial desynchronization of environmental fluctuation may cause stress with long-term, senescence-related consequences (Almaida-Pagán et al., 2018). While high levels of stress may induce immediate mortality, mild stress typically has life-extending effects (Gems and Partridge, 2008). Organisms that have evolved under chronic environmental fluctuation are expected to possess efficient stress-coping adaptations (Angilletta, 2009; Kern et al., 2015).

Temperature has a fundamental effect on lifespan and low body temperature is associated with longer lifespan in both ectotherms and endotherms (Flouris and Piantoni, 2015; Keil et al., 2015). In ectothermic vertebrates, chronic exposure to sub-optimal temperatures (i.e. temperatures outside the range of preferred body temperatures (Gvoždík, 2018)) has been studied particularly in fishes (Keil et al., 2015), confirming their role in lifespan extension. However, the effect on other life history traits was negative as a consequence of lower metabolism and slower development rate (Angilletta, 2009). The role of fluctuating temperatures on lifespan is less clear (Colinet et al., 2015). Fluctuating temperatures and their effect on lifespan have been studied primarily in invertebrates such as nematodes, crustaceans (*Daphnia* sp) or insects and variously demonstrated lifespan extension, reduction or no effect (Cedergreen et al., 2016; Colinet et al., 2015; Schwartz et al., 2016). The lifespan modulating effect of fluctuating temperature

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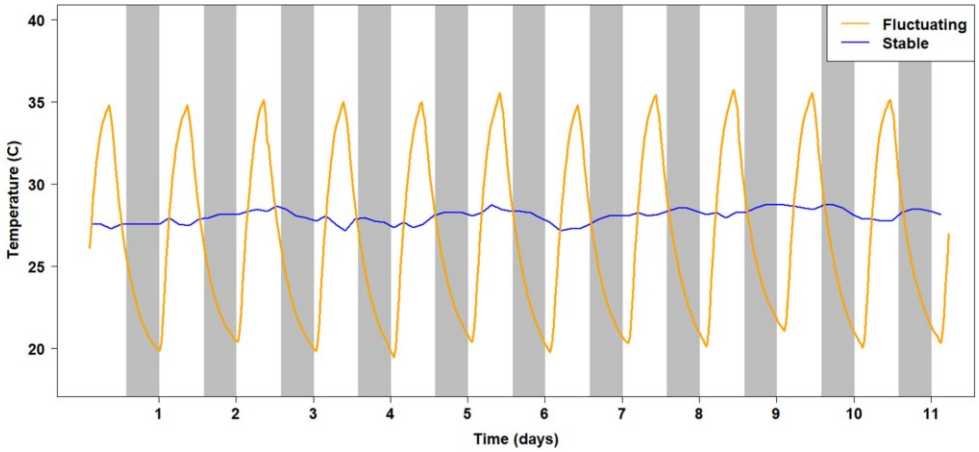


Fig. 1. Water temperature over a period of 11 days in stable (blue line) and fluctuating (orange line) temperature treatments. Grey areas represent the dark period of the day. A short time period was chosen for clear visualization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

remains largely untested in vertebrates, while both negative and positive effects of fluctuating temperature have been demonstrated on developmental rate (Kern et al., 2015; Niehaus et al., 2012), growth rate (Diana, 1984; Vondracek et al., 1988) and reproduction (Boulé and Fitzgerald, 1989; Podrabsky et al., 2008).

Growth and reproduction are commonly linked to survival via trade-off in resource allocation (Kirkwood, 1977; Lee et al., 2013), antagonistic pleiotropic genetic effects (Williams, 1957) or positive pleiotropic effects (Maklakov et al., 2015). From an evolutionary perspective, it is important to study growth and reproduction together with survival to obtain more complete information on the functional senescence of an organism (Lemaître and Gaillard, 2017; Monaghan et al., 2008). At least in insects, there appears to be no consistent link between survival and other life history traits across studies that exposed animals to fluctuating temperatures (reviewed in Colinet et al. (2015)). This is likely due to wide variation in the distance between the range of preferred body temperatures and the fluctuations tested, unpredictability of extrapolation from studies in stable temperature, and species-specific thermal sensitivity of traits examined (Colinet et al., 2015; Kingsolver et al., 2008; Niehaus et al., 2012). To our knowledge, the extent to which fluctuating temperatures contribute to modification of lifespan and its association with fundamental life history traits in vertebrates is unknown.

In the present study, we used the short-lived turquoise killifish *Nothobranchius furzeri*, a promising vertebrate model for aging (Hu and Brunet, 2018), to examine the role of fluctuating temperature on vertebrate lifespan and its relationship to thermally sensitive life history traits – growth and reproduction.

2. Material and methods

2.1. Study species

The turquoise killifish *Nothobranchius furzeri* is adapted to thermally challenging ephemeral savanna pools in south-east Africa and has a naturally short lifespan of several months (Reichard and Polačik, 2019). During the peak season of killifish abundance, water temperature in ephemeral savanna pools fluctuates in an asymptote of 15–20 °C daily, usually from 22 °C in the early morning to 38 °C in late afternoon (Žák et al., 2018). Perhaps as an adaptation to a thermally unstable environment, the turquoise killifish has wide range of preferred body temperature, from 20 to 30 °C (Žák et al., 2018). The species is easily bred in the laboratory under a stable temperature regime, with a recommended breeding temperature of 25–28 °C (Genade et al., 2005; Polačik et al., 2016). In the laboratory, lowering the water temperature from 25 °C to 22 °C extended the median lifespan of *N. furzeri* by 11% (from 9 to 10 weeks) and delayed the onset of molecular and behavioural markers of senescence (Milinkovitch et al., 2017; Valenzano et al., 2006). Nonetheless, laboratory studies used chronic exposure to lower temperature only, overlooking the fact that such conditions are unnatural for this species.

2.2. Fish maintenance

We used wild-derived population MZCS 222 (Cellerino et al., 2016) of turquoise killifish kept at the accredited breeding facility at the Institute of Vertebrate Biology of the Czech Academy of Sciences. Fish were hatched on 19 February 2018 following a standard breeding protocol (Polačik et al., 2016). During the first month after hatching, fish were kept under stable temperature of 27.5 °C ± 1 °C in two 60 L tanks. Fish were fed ad libitum 2–3 times per day, initially with live brine-shrimp nauplii and gradually (mixed feeding for 7 days) weaned onto frozen bloodworms (Chironomidae) as they grew larger.

At the age of 5 weeks (age at maturity of all individuals), 84 fish (40 females, 44 males) were individually housed in 2 L tanks within two independent recirculating systems (Aquamedic, Germany, www.aquamedic.de). Selection of experimental fish from initial stock was haphazard and fish from both original tanks were used across both treatments. Individually housed fish were fed ad libitum (amount consumed within 5 min) once per day between 9 and 10 am on thawed frozen bloodworms. Water conductivity was kept between 250 and 350 $\mu S \times cm^{-1}$ and the light regime was 14L:10D.

We used two treatments; stable temperature and fluctuating temperature, for a total period of 78 weeks. Stable temperature was maintained at 27.5 ± 1 °C (mean ± SD) throughout the experiment. Fluctuating temperature ranged from 20 °C ± 1 °C in early morning to 35 °C ± 1 °C in late afternoon (Fig. 1, Supplementary Fig. S1). The limits for fluctuating temperature were chosen to reflect the diurnal change in water temperature that killifish experience in the wild (Žák et al., 2018). The fluctuating temperature treatment was provided in one recirculating system by a combination of an aquarium chiller (TECO TR 10, Italy, www.tecoonline.com) and three aquarium heaters (2× 200 W and 1× 100 W, Eheim/Jäger, Wüstenrot, Germany). The second recirculating system was used for stable temperature (regulated by one 100 W heater, Eheim/Jäger, Wüstenrot, Germany). One temperature datalogger (HOBO Onset Computer, UA-002-64, Bourne, MA, USA) monitored temperature in the middle rack of each recirculating system. The consistency of thermal conditions and water parameters was checked every two weeks and the set-up of chiller or heaters was modified as necessary to maintain the same thermal regime irrespective of minor seasonal changes in room temperature over the 78 week duration of the experiment (room temperature ranged between 25 °C and 29 °C).

2.3. Data collection

Fish health status and survival were monitored daily. Fish body size was measured every week from the age of 29 days to the age of 300 days and every two weeks until the age of 466 days, using photographs taken in a plastic container with shallow water and a scale on the bottom (resolution of 1 mm). Photographs were imported to ImageJ v 1.46 (imagej.nih.gov) and body size was measured as Standard Length (from tip of the snout to the end of caudal peduncle). Individual specific growth rate (SGR, % SL per day) was estimated from the first 30 days of the experiment, when growth rate was highest and linear (Blažek et al., 2013, Fig. 4). The SGR was calculated as $(\ln SL_2 - \ln SL_1) \times (100/t)$ where SL_2 is terminal body length, SL_1 is initial body length and t is the length of time interval in days (Talbot, 1993).

Reproductive parameters were estimated monthly from spawnings in 2 L plastic containers with a 5 mm layer of fine sand as a spawning substrate. A pair of fish was placed in the container and allowed to spawn for 2 h, which is sufficient for release of all ovulated eggs from female ovaries (Polačik et al., 2016). The same pairings were used throughout the study, though when one individual from a pair died, it was replaced by another fish from the same treatment that lost its partner. At time of spawning termination, all pairs were moved back to recirculating system in a haphazard mode (alternating pairs from stable and fluctuating temperature) within 25 min. The periodicity of spawning was every week but eggs were counted for analysis only once per month. Weekly spawnings were performed to prevent egg retention in ovaries (Polačik et al., 2014), a common problem in female *N. furzeri* when housed in isolation (Polačik et al., 2016). In the present experiment, one female (4.5%) from stable temperature and three (13.6%) females from fluctuating temperature were suspected to have died due to inability to release their eggs (a significant reduction from 80% reported by (Polačik et al., 2014)). Those fish were omitted from the analysis of reproductive traits. The total number of eggs (fecundity; proxy of female reproductive output), number of fertilized eggs (fertility; proxy of reproductive fitness) and fertilization rate (proportion of fertilized eggs; proxy of oocyte quality; in *N. furzeri* fertilization rate is dependent on quality of eggs and almost independent of male traits, Polačik et al., 2016) were determined by sieving eggs from the sand 24 h after removal of parental fish from containers. Fertilized eggs were determined by the presence of the perivitelline space (Polačik et al., 2016).

2.4. Statistical analysis

Median lifespan was compared between treatments using a non-parametric Log-rank test, including sex-specific response. All survival curves were visualized by Kaplan-Meier plots.

The association between lifespan and other life history traits was analysed using a sex-specific Gamma Generalized linear model (GLM) with a log-link function. Sex-specific models were used because reproductive parameters - early fecundity (number of eggs after 30 days under each thermal regime) and fertilization rate (maximum fertilization rate achieved by a female) - were only recorded for females. Early fecundity, fertilization rate, thermal regime and body length at 59 days (period including both juvenile growth and the effect of thermal regime on growth) were included as predictors of female lifespan. SGR was not used for females, because it was collinear with body length (variance inflation factor > 4). For males, lifespan was modelled as a function of thermal regime, body length at 59 days and SGR during the first month of the experiment. In both models for males and females, all possible two-way interactions were included in the full model and removed when insignificant. Given a significant interaction between treatment and body size in males (Supplementary Table S2), thermal regime-specific analyses were conducted.

In addition, as an evaluation of disposable soma theory, we compared how the relationship between lifetime fecundity and female lifespan varies between thermal regimes using Gamma GLM with lifespan as a response variable and lifetime fecundity in interaction with thermal regime as explanatory variables.

The growth trajectory was compared between thermal regimes with a sex-specific Gaussian Generalized Additive Model (GAM). Body size was the response variable, age was a smoothed predictor with the basis dimension 10 (k = 10) and thermal regime specified smoother (by = thermal regime). Thermal regime was added as a parametric predictor and Individual ID was used as a random factor. Specific growth rate from the first month in the experiment was compared using a GLM with SGR as a response variable and thermal regime in interaction with sex used as predictors. We also compared initial body size of fish between thermal regimes using a GLM with thermal regime and sex as explanatory variables.

Fecundity was analysed using a negative-binomial GAM with the number of eggs as a response variable and age (continuous, in days) as a smoothed parameter (k = 7). The addition of thermal regime-specific smoothers was not needed (see Results). Parametric terms were thermal regime and body size. Female ID was specified as a random factor. The same model structure was used for fertility analysis, with number of fertilized eggs used as a response variable instead of total number of eggs.

Fertilization rate was analysed with a binomial GAM, with raw binomial data as a response variable (number of fertilized eggs, number of unfertilized eggs), age as a smoothed predictor (k = 5, with k chosen on the basis of superior model fit, as estimated by AIC) and thermal regime as a parametric predictor. Female ID was specified as a random factor. The model was accounted for data overdispersion.

All procedures were performed in R 3.6.1. environment using *survival* 2.44.1.1, *lme4* 1.1.21, *mgcv* 1.8.28, *splines* 3.6.1, *car* 3.0.4 and *effects* 4.1.3 packages (Bates et al., 2015; Fox and Weisberg, 2019; R Core Team, 2019; Therneau and Grambsch, 2000; Wood, 2017).

3. Results

3.1. Effect of thermal regime on lifespan

Fish in fluctuating temperatures had a 93% longer median lifespan than fish kept in stable temperature (log-rank, $\chi_1^2 = 6.3$, $p = 0.010$, $N = 84$, Fig. 2a). A longer median lifespan in fluctuating temperatures was recorded in males ($\chi_1^2 = 5.4$, $p = 0.020$, $N = 44$) but this difference, although numerically larger, was non-significant in females ($\chi_1^2 = 1.5$, $p = 0.200$, $N = 40$, Fig. 2b). Overall, median lifespan did not differ significantly between males and females within thermal regimes (fluctuating: $\chi_1^2 = 3.5$, $p = 0.06$, $N = 42$; stable: $\chi_1^2 = 0$, $p = 0.90$, N = 42).

3.2. Effect of thermal regime on lifespan correlates

In females, thermal regime did not affect the relationship between lifespan and fundamental life history traits. A positive association between lifespan and fertilization rate ($\chi_1^2 = 9.23$, $p = 0.002$, Supplementary Table S1, Fig. 3a) was congruent across both thermal regimes ($\chi_1^2 = 1.41$, $p = 0.234$, $N = 35$). There was no effect of body size, early fecundity or their interaction with thermal regime on female lifespan (Fig. 3a, Supplementary Table S1).

There was positive relationship between lifespan and lifetime fecundity in females, with steeper increase detected in stable than in fluctuating temperature (Gamma GLM, lifetime fecundity:thermal regime interaction, $\chi_1^2 = 10.84$, $p = 0.001$, N = 35, Supplementary Fig. S2, Table S2).

In males, a positive relationship between lifespan and body size was detected in stable temperature ($\chi_1^2 = 5.01$, $p = 0.025$, $N = 21$) but not in fluctuating temperature ($\chi_1^2 = 0.022$, $p = 0.873$, N = 21, Fig. 3b, Supplementary Tables S3, S4). There was no effect of SGR on male lifespan (Supplementary Tables S3, S4).

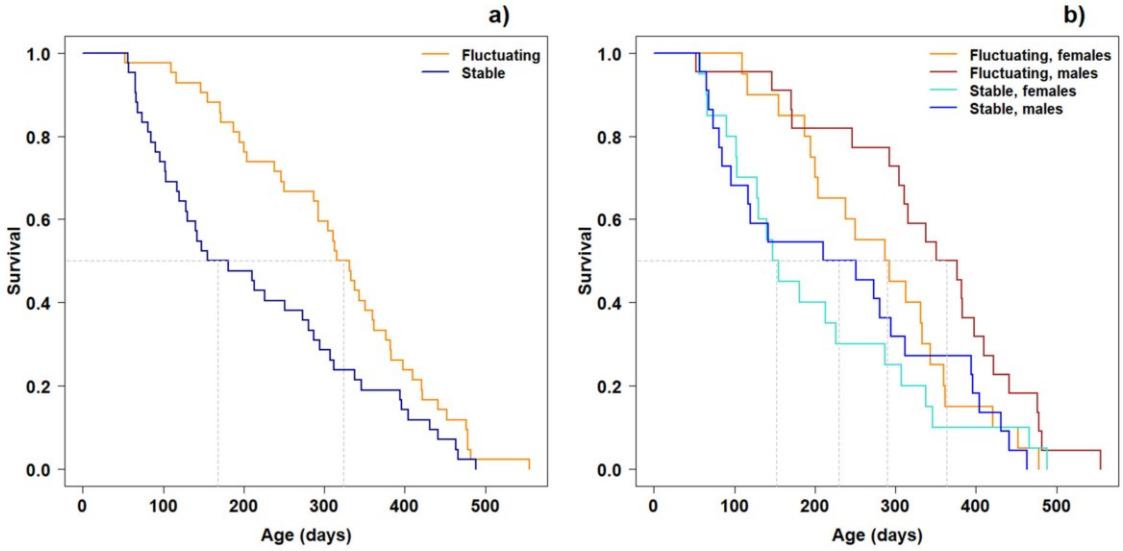


Fig. 2. Different survival of *Nothobranchius furzeri* in stable (blue, $N = 42$) temperature and in fluctuating (orange, N = 42) temperature a). Survival was longer in fluctuating temperature ($p = 0.010$). Sex and thermal regime specific survival of *Nothobranchius furzeri* b). Both survival plots are Kaplan-Meier plots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Effect of thermal regime on growth

Overall, fish in fluctuating temperature were smaller than fish in stable temperature (Gaussian GAM, males: $F_{1,1008} = 7.08$, $p = 0.009$, $N_1 = 44$ fish, $N_2 = 1066$ measurements; females: $F_{1,973} = 41.33$, $p < 0.001$, $N_1 = 40$, $N_2 = 1036$; Fig. 4, Supplementary Table S5). After the first month, female SGR was 53% lower in fluctuating temperature than in stable temperature. In males, the reduction in SGR between fluctuating and stable temperatures was 20% (thermal regime:sex: ANOVA, $F_{1,77} = 5.31$, $p = 0.024$, $N = 84$, Supplementary Table S6). At the beginning of the experiment, fish in both thermal regimes had similar body size (treatment: $F_{1,82} = 0.196$, $p = 0.660$, N = 84, Supplementary Table S7).

3.4. Effect of thermal regime on reproductive traits

Relative fecundity was 80% higher in females in fluctuating temperature (Neg.Bin. GAM, $\chi_1^2 = 7.93$, $p = 0.005$, $N_1 = 35$, $N_2 = 223$; corrected for female body size: $\chi_1^2 = 8.68$, $p = 0.003$). Females in fluctuating temperature effectively compensated their egg production despite their smaller size and, overall, there was no significant difference in absolute fecundity between thermal regimes ($\chi_1^2 = 0.74$, $p = 0.391$). The age-related pattern of relative fecundity (age: edf = 3.736, $\chi^2 = 127.19$, $p < 0.001$; Fig. 5a) was similar in both thermal regimes (model with treatment-specified smoothers AIC = 1836, without treatment-specified smoother AIC = 1832). Likewise, fertility did not differ between the treatments (Supplementary Table S8). There was no significant difference in fertilization rate between thermal regimes (Binomial GAM: $F_{1,177} = 0.188$, $p = 0.665$;

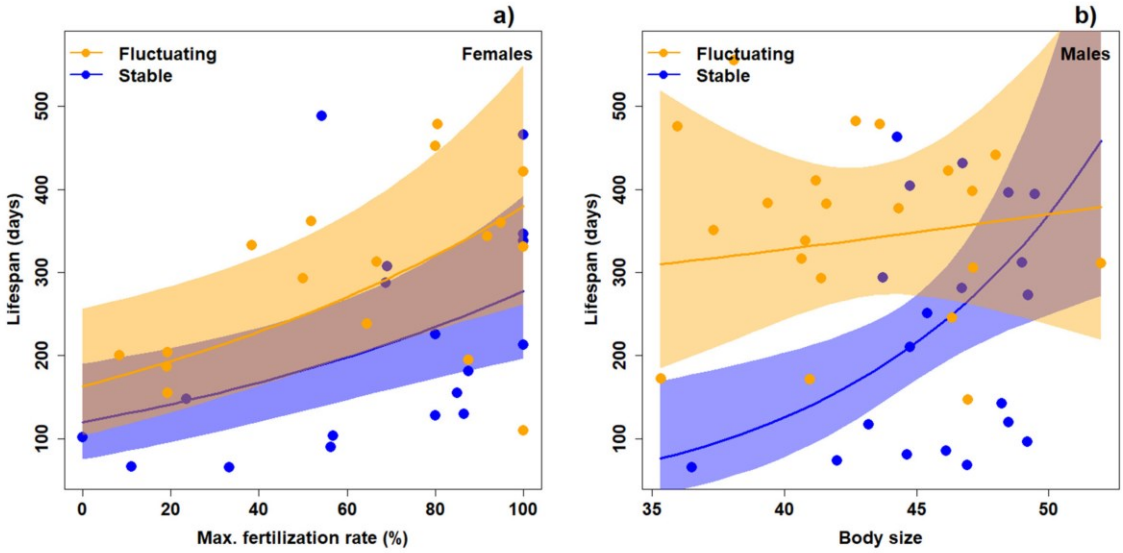


Fig. 3. Life history traits with significant sex-specific association with lifespan. (a) Positive association between fertilization rate and lifespan in females ($p = 0.002$, $N = 35$). (b) Treatment-specific association between body size and lifespan in males (stable: $p = 0.025$, $N = 21$; fluctuating: $p = 0.873$, N = 21). The curves were fitted by Gamma Generalized linear models. Shaded areas represent 95% confidence intervals. Filled circles denote raw data.

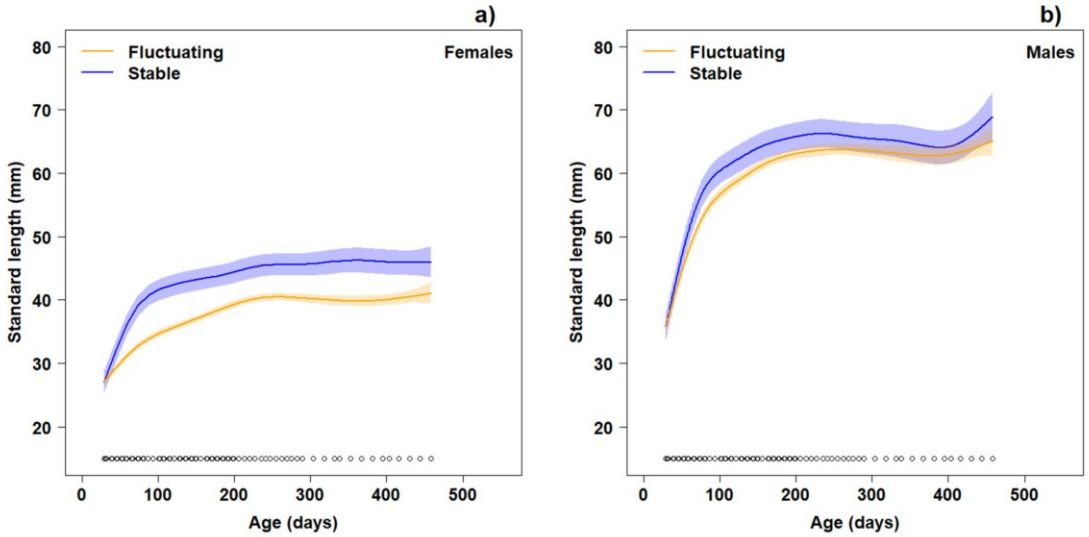


Fig. 4. Growth of (a) females ($p < 0.001$, $N = 40$) and (b) males ($p = 0.009$, $N = 44$) was higher in stable than in fluctuating temperature. The curves were fitted by Gaussian Generalized Additive Models with specified treatment-specific smoothing factor. The lines represent means and shaded areas are 95% confidence intervals. Empty circles at the bottom of plots represent time-points when body-size measurements were taken. Due to age-related dying (Fig. 2) sample size was decreasing with age.

$N_1 = 35$, $N_2 = 209$); the data demonstrate a decrease in fertilization rates in both treatments, followed by a short terminal increase caused by the longest surviving females (edf = 3.852, F = 8.291, p < 0.001, Fig. 5b). A treatment-specific smoother for age was not needed (ΔAIC = 9).

4. Discussion

Previous studies have detected a detrimental effect of stable temperature on early development of ectothermic vertebrates (Ashmore and Janzen, 2003; Niehaus et al., 2012). Using the short-lived fish *Nothobranchius furzeri*, we demonstrated that stable temperature shortened lifespan and decreased relative fecundity, but increased growth rate compared to individuals experiencing fluctuating temperatures.

Chronic exposure to stable temperature shortened the median lifespan of turquoise killifish despite the fact that fish in fluctuating temperatures spend approximately a quarter of the day in temperatures 5–6 °C above the upper range of their preferred body temperatures (i.e. 30 °C (Žák et al., 2018)). Jensen's inequality, validated on stable temperatures, postulates that organismal performance is disproportionally more sensitive to warm temperatures than to low temperatures (Martin and Huey, 2008). Thus, it is predicted that the upper limit would have a disproportionately greater (i.e. lifespan-shortening) effect. Nonetheless the effect of fluctuating temperature on life history traits appears to be contingent upon the range of experimental temperatures and their distance from optimal temperatures (Colinet et al., 2015; Kingsolver

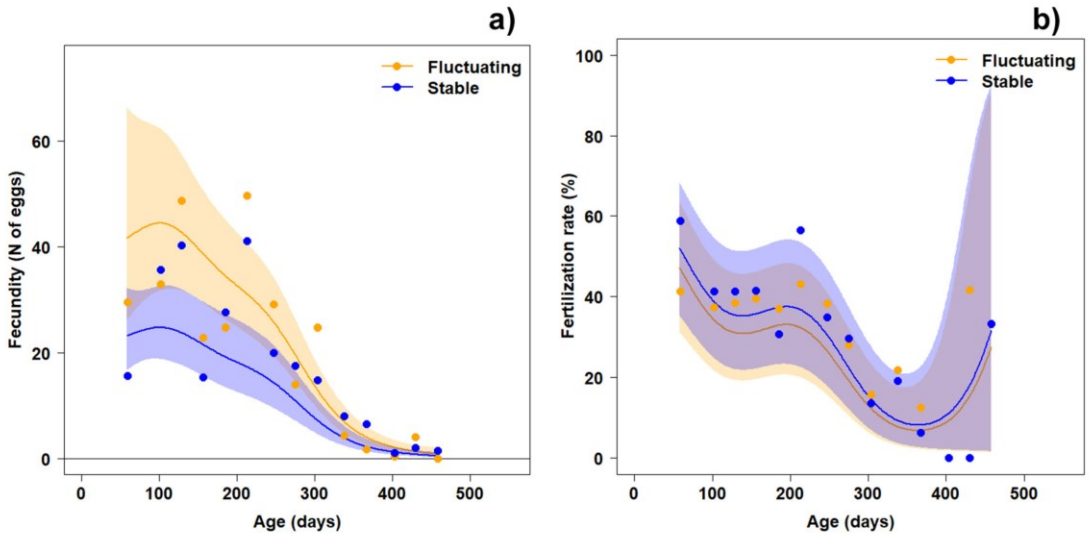


Fig. 5. Age-related patterns of (a) fecundity and (b) fertilization rate of females (N = 35) kept in stable (blue) and fluctuating (orange) temperature. Fecundity (corrected for body size) was analysed using negative-binomial Generalized additive model (GAM) and was higher in fluctuating temperature treatment ($p = 0.005$). The fertilization rate was fitted by binomial GAM and similar in both regimens ($p = 0.665$). Circles represent mean values computed from raw data. Note that empty circles do not necessarily follow the curve due to random factor effects and, for fecundity estimates, correction for body size. Due to age-related mortality (Fig. 2), sample size was decreasing with age. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2008). We fluctuated the temperature between 20 °C and 34 °C, which reflects conditions commonly experienced by turquoise killifish in the wild (Reichard et al., 2009; Žák et al., 2018). The length of time the killifish experienced these temperatures was probably too short and/or temperatures too close to the optimal range to have a detrimental effect on lifespan. Close proximity to optimal temperatures is supported by the fact that the upper range of fluctuating temperatures was 7 °C below the lethal critical thermal maximum for this species (Philippe et al., 2018). Therefore, it is reasonable to suggest that turquoise killifish possess efficient physiological mechanisms to cope with temperatures 5–6 °C above the upper limit of their preferred body temperatures.

The observed increase in lifespan in fluctuating temperature may have been induced by a stress-protective response (Gems and Partridge, 2008; Judy et al., 2013; Sorensen et al., 2003). Thermal stress increases production of reactive oxygen species and induces up-regulation of heat-shock proteins (Hsps) (Banh et al., 2016; Podrabsky et al., 2008). It is assumed that metabolism and oxygen consumption during the warm phase of fluctuation is higher than at a similarly warm stable temperature (Beauregard et al., 2013; Lyytikainen and Jobling, 1998; Martin and Huey, 2008). This may induce mild stress in the form of dietary restriction which may contribute to lifespan extension (Schwartz et al., 2016). However, it is unlikely the case in our study, as dietary restriction commonly reduces the fecundity (Vrtílek and Reichard, 2015) while we observed relative increase in fecundity. Higher metabolic rate in fluctuating temperature contributes to detrimental oxidative stress and hence accelerates functional senescence (Banh et al., 2016; Kregel and Zhang, 2007). Hsps are a large group of proteins with various effects on lifespan and life-history traits when up-regulated (Lithgow, 1996; Sorensen et al., 2003). They are able to repair protein damage induced by both thermal and oxidative stresses and they are a candidate mechanism for why we observed longer lifespan in fluctuating temperature (Lithgow, 1996; Sorensen et al., 2003). The association of specific Hsp and lifespan in turquoise killifish is unknown, but previous work on the Neotropical annual killifish *Austrofundulus limneus* showed that fluctuating temperature up-regulated small Hsps (Hsp22, Hsp27) while stable temperature conditions led to the up-regulation of large Hsps (Hsp70, Hsp90) (Podrabsky and Somero, 2004). We believe that it is reasonable to assume that up-regulation of different Hsps may contribute to the observed difference in lifespan between thermal regimes. Further, the activation of other stress protective mechanisms such as progranulin pathway may also play a role (Judy et al., 2013).

Fish in fluctuating temperature experienced slower growth and reached smaller body size. The temperature-size rule postulates that, in fish, oxygen supply is more important for anabolism than food supply (Forster et al., 2012; Pauly, 1981) and oxygen availability for tissues is lower at higher temperatures. Hence, fish experiencing warmer temperatures reach smaller sizes, especially among tropical fish species (Audzijonyte et al., 2020). We believe that the main mechanism behind the smaller body size of fish in fluctuating temperature is the combination of periodic cold exposure and an increase in mandatory energy consumption higher than the maximum metabolic rate during warm periods, reducing the amount of available energy to growth (Gvoždík and Kristín, 2017; Kingsolver and Gomulkiewicz, 2003; Lyytikainen and Jobling, 1998). Alternatively, smaller asymptotic body size in fluctuating temperatures could be explained by the cost of repairing mechanisms (Sorensen et al., 2003) or by smaller cell size (Adrian et al., 2016). Irrespective of the mechanism, fluctuating temperature constrained the maximum body size of turquoise killifish.

Slower growth rate is often associated with longer lifespan (Lee et al., 2013). This relationship held for the gross contrast between treatments, with fish in fluctuating temperature reaching smaller body size and living longer. At the individual level, the same association was demonstrated for males in the stable thermal regime, as reported in previous studies (Baumgart et al., 2016; Kirschner et al., 2012). However, the relationship did not hold for males in fluctuating temperature, indicating that extended male lifespan in fluctuating temperature is not causally linked to decreased growth rate or smaller body size. This illustrates that the assumptions derived under stable temperatures are of limited utility for predicting organismal performance under ecologically relevant temperature fluctuations (Colinet et al., 2015; Niehaus et al., 2012).

Females experiencing fluctuating temperature were more fecund, relative to their body size. Even when fecundity was not corrected for body size, their fecundity was similar to females at stable temperature despite their smaller body size. Increased thermal fluctuation may be a signal of water level decrease and thus induce higher investment to current reproduction, as reported for *N. wattersi* under experimental conditions (Grégoir et al., 2017). Surprisingly, Podrabsky et al. (2008) found that fluctuating temperature had a negative (rather than positive) effect on fecundity and fertilization rate in the Neotropical annual killifish *Austrofundulus limnaeus*, despite the fact that *A. limnaeus* in the wild experience fluctuating temperatures closely resembling the thermal dynamics in ephemeral pools inhabited by the turquoise killifish (Podrabsky et al., 1998; Žák et al., 2018). Inconsistent findings between our study and that of Podrabsky et al. (2008) may be due to the different range of temperatures used, the period for which fish were subjected to fluctuating temperature (which was only 2 months in Podrabsky et al., 2008), the effect of different food rations (fish fed twice per day in Podrabsky et al., 2008) or the different thermal requirements of the two species.

The thermal regime did not affect patterns of reproductive senescence in fecundity, fertilization rate or fertility despite the fact that females in fluctuating temperature tended to have longer lifespans. Across both treatments, females with higher fertilization rates lived longer and early fecundity was not negatively associated with individual lifespan. Overall, there was positive relationship between female lifespan and lifetime fecundity in both thermal regimes (despite slope of the relationship was thermal-regime specific) which indicates that short-lived females did not compensate their fecundity to shorter lifespan. This is in contrast to the trade-off assumptions of the disposable soma theory of senescence (Kirkwood, 1977) which assume that high investment in early reproduction has costly consequences expressed later in life. In that respect, our data support the predictions of positive pleiotropy where “good quality” individuals possess superior fitness traits (at least in a given environment), including reproductive outcome and survival (Maklakov et al., 2015).

Our findings demonstrate that the expression of life history traits and their associations observed under stable temperature are a poor representation of relationships arising in the fluctuating thermal conditions of natural habitats. The lifespan of *N. furzeri* males was significantly extended under fluctuating temperature and the same trend was present in females. Females in fluctuating temperature had higher fecundity, with no detrimental effects on reproductive senescence or survival, suggesting a lack of allocation trade-offs or negative pleiotropy. Overall, our results have important consequences for future laboratory studies on functional and actuarial senescence, as most laboratory studies are conducted in stable experimental temperature. Future studies should study the physiological and molecular mechanisms underlying lifespan and life history differences in *N. furzeri* exposed to different thermal regimes.

CRediT authorship contribution statement

Conceptualization: JZ, MR.
Data curation: JZ, MR.
Formal analysis: JZ.
Funding acquisition: MR, JZ.
Investigation: JZ.
Methodology: JZ, MR.
Project administration: MR, JZ.

Resources: MR.
Supervision: MR.
Validation: JZ, MR.
Visualization: JZ.
Writing - original draft: JZ, MR.
Writing - review & editing: JZ, MR.

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Data availability

Data and R code for the analyses are available at Figshare (doi: https://doi.org/10.6084/m9.figshare.12546632).

Declaration of competing interest

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2020.111073.

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PAPER 10: Replacement of live food with pellets

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Good performance of turquoise killifish (*Nothobranchius furzeri*) on pelleted diet as a step towards husbandry standardization

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Dietary alteration is one of the most universally effective aging interventions, making its standardization a fundamental need for model organisms in aging. In this dietetic study we address the current lack of standardized formulated diet for turquoise killifish *Nothobranchius furzeri* – a promising model organism. We first demonstrated that *N. furzeri* can be fully weaned at the onset of puberty onto a commercially available pelleted diet as the sole nutrition when kept in social tanks. We then compared nine somatic and six reproductive parameters between fish fed a typical laboratory diet - frozen chironomid larvae (bloodworms) and fish weaned from bloodworms to BioMar pellets. Both dietary groups had comparable somatic and reproductive performance. There was no difference between diet groups in adult body size, specific growth rate, condition or extent of hepatocellular vacuolation. Fish fed a pelleted diet had higher juvenile body mass and more visceral fat. Pellet-fed males had lower liver mass and possessed a lipid type of hepatocellular vacuolation instead of the prevailing glycogen-like vacuolation in the bloodworm-fed group. No considerable effect was found on reproductive parameters. The negligible differences between dietary groups and good acceptance of pellets indicate their suitability as a useful starting point for the development of standardized diet for *Nothobranchius furzeri*.

A standardized diet is an important prerequisite in studies of the mechanisms underlying the biological phenomenon of aging[1]. Hence there is a high demand for the standardization of laboratory diets[2,3]. Feeding laboratory organisms live food and food of wild origin has numerous drawbacks such as the risk of disease introduction[4], chemical contamination of food affecting physiology[5], seasonal availability or instability of nutritional content[6] and high waste production[4]. All these problems can be avoided by a standardized pelleted diet. Moreover, experimental studies benefit from easy manipulation of the nutritional content of the formulated diet and its effect on aging, lifespan, growth or reproduction[7,8]. A standardized diet is sometimes not available for established model organism, such as *Danio rerio*[9,10] or organisms newly introduced to the laboratory culture, such as the short-lived turquoise killifish *Nothobranchius furzeri*[2,3]. The development of standardized diet for *N. furzeri* is hampered by perceived reluctance of this fish to accept dry food[2]. The absence of a standardized diet likely impedes wider use of turquoise killifish as a laboratory model[11].

Diet has a profound effect on survival and aging of experimental animals and it is one of the key components in aging intervention studies[1,8]. Diet affects lifespan probably via a maintenance-reproduction trade-off[12] and hence there is a need to understand the effect of diet on a wide range of life history traits. Indeed, the type of diet has different effects on life history in many fish species. For example in zebrafish *Danio rerio* and Siamese fighting fish *Betta splendens*, growth, condition, fecundity and fertilization rate vary between live food and a pelleted diet[13,14]. The specific diet fed to parents can often affect their offspring[15,16]. The impact of diet on life history outcomes thus presents an excellent measure for testing the effect of a pelleted diet on *N. furzeri* compared to the commonly used frozen bloodworms (*Chironomus* larvae)[2].

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	[a] INICIO 0.4 mm	[a] INICIO 0.6 mm	[a] INICIO 0.8 mm	[a] INICIO 1 mm	[a] INICIO 1.5 mm	[b] Bloodworms	[b] INICIO 0.8 mm
Crude protein	63.0%	62.0%	56.0%	56.0%	54.0%	53.8% ± 3.0%	59.5% ± 3.0%
Crude lipid	11.0%	13.0%	18.0%	18.0%	22.0%	5.2% ± 8.0%	17.2% ± 8.0%
Carbohydrates (NFE)	7.3%	6.3%	9.0%	9.0%	7.7%	22.8% ± 20.0%	11.0% ± 20.0%
Ash	12.5%	12.5%	11.5%	11.5%	11.0%	18.1% ± 2.5%	12.3% ± 2.5%
Moisture	—	—	—	—	—	82.9% ± 2.0%	5.9% ± 2.0%

Table 1. Macronutrient composition of diets fed to turquoise killifish. [a]Official declaration of the manufacturer - BioMar INICIO, Denmark. [b]Values from our analysis. Percentages of Crude protein, Crude lipid, Carbohydrates (NFE) and Ash are computed after subtraction of moisture. Values behind ± are precision of the analytical measurements. NFE stands for nitrogen free extract.

Diet usually influences storage of energetic reserves in livers[17]. On the cellular level, these energetic reserves appear as vacuoles in hepatocytes filled with glycogen and/or lipids[17]. In general, fish tend to accumulate more glycogen/lipids and, consequently, have larger vacuoles than mammals[18]. In mammals, such extent of hepatocellular vacuolation would be considered as lipidosis or steatosis[19]. Findings from wild annual killifish suggest that high hepatocellular vacuolation is a natural phenomenon[20,21] which is understandable for a species with high energy demands on growth and reproduction in the unpredictable environment of ephemeral pools[11]. There is no strong empirical evidence that highly vacuolated livers with compact cellular membranes are not functioning properly[19], though the degree of vacuolation when the hepatocellular membrane disintegrates and neighboring cells fuse is suggested to be pathological condition in the majority of fish species[18]. Liver histopathology is one of the most important markers in *Nothobranchius* aging studies[22,23] and it is necessary to provide baseline data for the introduction of a new diet.

Nothobranchius furzeri is an important vertebrate model in biomedical and evolutionary studies on aging[11,24,25]. It has an unprecedented fast life history adapted to shallow ephemeral savanna pools in south-east Africa[11] but it can be easily bred in captivity[2,3]. It is a short-lived vertebrate[26], with a lifespan of a 1-5 months in the wild[27] and 3-16 months in captivity[26,28]. Their eggs are desiccation resistant and can be stored in substrate for months to years under laboratory conditions[2,11]. This invertebrate-like characteristics and vertebrate-like body plan makes them ideal organism for laboratory studies[11,24]. Natural diet of *N. furzeri* consists of small aquatic invertebrates[29]. In the laboratory, dietary restriction[23] and supplementation with antioxidant resveratrol[30] has been shown to extend the lifespan of *N. furzeri*, demonstrating the substantial role of dietary manipulations. Unfortunately, the absence of a standardized diet limits the validation of experimental results across different laboratories[2,3].

At present, the typical laboratory diet fed to *Nothobranchius* spp. consists of frozen bloodworms (larvae of Chironomidae) but there is considerable variation in the quality of bloodworms from different commercial suppliers and consequently among different laboratories[2,3]. Less common forms of diet used for adult nothobranchids include gelatin cubes made of bloodworms[30], freeze-dried bloodworms[31,32], *Tubifex* sp. worms[33], *Chaoborus* larvae[25] or a combination of *Artemia nauplii* and flake food[34]. Undoubtedly, the above mentioned studies would have benefited from the existence of a standardized diet. To our knowledge, there have been several unpublished unsuccessful attempts to adapt *N. furzeri* to pelleted diet. Only one work was at least partially successful but still co-feeding with live *Artemia* was necessary[35]. This is because *Nothobranchius* spp. strongly prefer natural food and usually avoid the common, commercial dry or gelatin-like fish foods accepted by many other fish models.

In the present study, we used a wild-derived strain of *N. furzeri* (MZCS 222) kept in social groups to investigate their ability to accept the commercially available dry pellets BioMar INICIO. This formula was developed to meet the nutritional requirements of juvenile salmonids, which are among the most important cultured fishes globally. After successful conversion of fish to dry pellets, we compared key fitness-related life history traits between the pellet-fed diet group and a bloodworm-fed control. We compared growth, Fulton's condition factor, visceral fat score, liver mass, liver histology, fecundity, fertilization rate, reproductive allotment, egg size, egg survival and egg development dynamics between the two dietary groups. We believe that the present study makes a substantial contribution to the development of the unified laboratory diet for this important model organism.

Results

All fish selected for the pellet diet treatment were successfully weaned from *Artemia* nauplii and bloodworms to pellets between the age of 12 and 21 dph (days post hatching), with most fish weaned by 17 dph. Pellet food had a higher lipid content (11–22% vs. 5%) while bloodworms were richer in carbohydrates (23% vs 6–9%). The detailed macronutrient content of the diets is provided in Table 1.

Somatic parameters. The dietary groups differed in four of the nine somatic parameters we measured (Figs. 1 and 2). Over 35 days of either the pellet or bloodworm diet (corresponding to the period of most intense growth) fish increased their body mass, on average, from 0.096 g of juvenile mass to 1.203 g in females and 2.547 g in males (Fig. 1a).

Individual body size did not differ between treatments at start of the experiment at 15 dph (Linear mixed effect model, LME: $P = 0.196$), at the end of juvenile period at 30 dph (males: $P = 0.280$, females: $P = 0.287$), nor at the end of the experiment at 56 dph (males: $P = 0.973$, females: $P = 0.751$, Fig. 1b). Initial body mass was higher in the pellet diet treatment at age 15 dph: 19% difference, $P = 0.003$ and this difference increased considerably by the end of the juvenile period (age 30 dph: males: 80%, $P = 0.012$, females: 28%, $P = 0.009$, Fig. 1a). At the end of

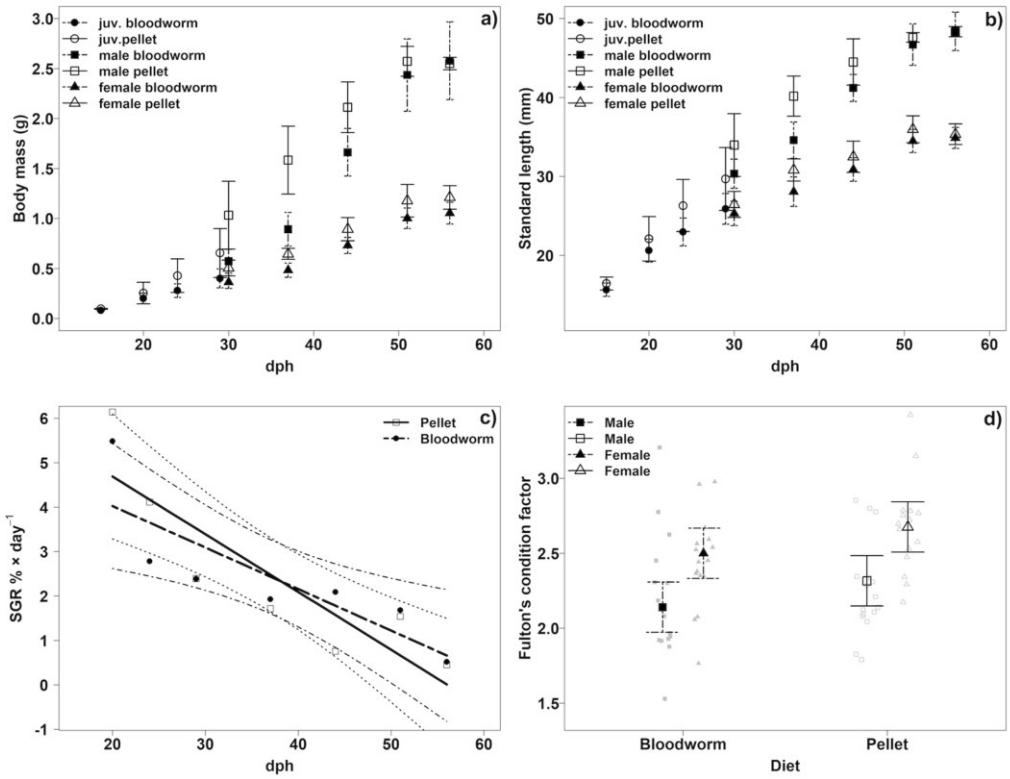


Figure 1. Growth and condition parameters in the two dietary groups. (**a**) Age-dependent body mass. (**b**) Age-dependent standard length. Values in a) and b) for juveniles are tank dependent weighted means (weighted by number of fish). Confidence intervals (CI, 95%) are computed from tank-specific weighted means. (**c**) Specific growth rate of body size in % per day during whole experimental period in both diet groups. Linear model fit with 95% CI. SGR for body mass is not shown given its similarity with body mass SGR. (**d**) Fulton's condition factor with raw data points and model estimated means with 95% CI.

the experiment, there was no difference in male body mass (age 56 dph: $P = 0.920$) and only a minor difference in female body mass (15%; $P = 0.017$, Fig. 1a). The specific growth rate over the entire experimental period was similar in both groups (Linear regression, body size: $P = 0.949$, body mass: $P = 0.960$; Fig. 1c). Similar growth rates in both dietary treatments were accomplished by a higher consumption of bloodworms compared to pellets (Supplementary Fig. S1). This resulted in a food conversion ratio (FCR) of 7.44 (range 5.39-10.36) for bloodworms and 0.99 (0.67-1.93) for pellets.

Body mass differences between dietary treatments could be related to body condition but Fulton's condition factor was similar between the treatments (7% difference, LME, $P = 0.0503$, Fig. 1d). Visceral fat score was higher in the pellet-diet treatment (males: 27%, Multistratum permutation analysis, $P = 0.015$; females, 87%, $P < 0.001$, Fig. 2a). A sex-specific effect of diet on liver mass was detected. Liver mass controlled for eviscerated body mass did not depend on diet in females (7%, LME, $P = 0.0531$, Fig. 2b) while bloodworm-fed males had 42% higher liver mass than pellet-fed males ($P < 0.001$, Fig. 2b).

Histological examination of the liver parenchyma revealed two essential types of hepatocellular vacuolation (Fig. 3). (i) The lipid type manifested itself as unstained cytoplasmic vesicles with sharp edges. (ii) The glycogen-like type was characterized by irregular vacuoles containing slightly flocculent material. The presence of glycogen was evidenced by periodic acid Schiff reaction (PAS) positive staining of unevenly sized grains dispersed in the cytoplasm (Fig. 3e). Fish fed pellets had almost exclusively lipid type hepatocellular vacuolation while fish fed bloodworms had mostly the glycogen-like type (χ^2 test, $P = 0.003$, Figs. 2d and 3). Out of 41 histologically examined fish, only a single female (fed bloodworms) had no sign of hepatocellular vacuolation (Fig. 3a). Hepatocellular vacuolation extent was similar in both treatments (LME, $P = 0.838$, Fig. 2c) but males were more affected than females ($P < 0.001$). Male livers with glycogen-like type vacuolation were larger than livers with lipid type vacuolation (54%, LME, $P < 0.001$, Supplementary Fig. S2) but male liver mass was independent of the extent of hepatocellular vacuolation ($P = 0.461$). In contrast, female liver mass was independent on vacuolation type ($P = 0.260$) but depended on vacuolation extent ($P < 0.001$, Supplementary Fig. S2). Pre-neoplastic lesions were found only in one fish fed bloodworms and three fed pellets, too few for statistical comparison.

Reproductive parameters. While fish reproductive parameters typically depend on diet[8], none of the six reproductive parameters we measured differed between the treatments (Fig. 4). Therefore, egg number (Negative binomial Generalized linear mixed effect model (GLMM), $P = 0.242$, Fig. 4a), fertilization rate (Binomial GLMM,

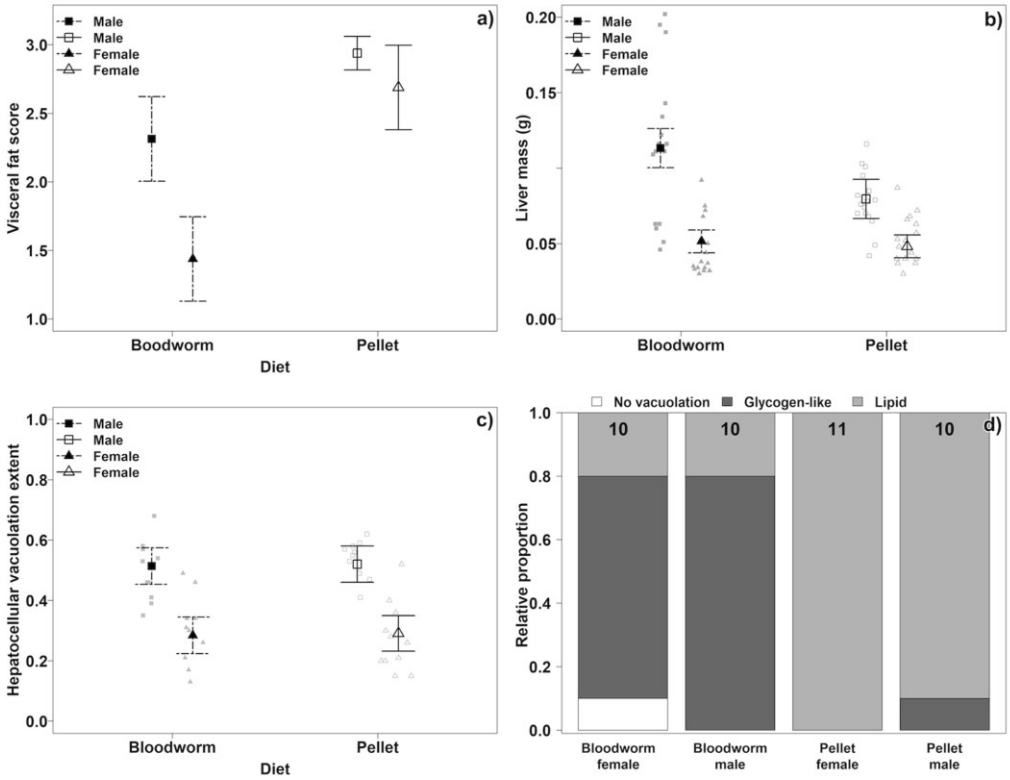


Figure 2. Somatic parameters in the bloodworm-fed and pellet-fed dietary groups. (**a**) Visceral fat score. The values represent sex and tank dependent means and their associated CIs. (**b**) Liver mass corrected for eviscerated body mass. Model estimated means with 95% CI and original data points. (**c**) Relative hepatocyte cytoplasmic vacuolation. Model estimated means with 95% CI and original data points. (**d**) Proportion of hepatocyte cytoplasmic vacuolation types computed from raw data. Numbers in upper parts of bars indicate sample sizes. Note that observation points in plots do not necessarily fit to the presented means due to the role of random factors. Figures without observation points were based on tank-specific means.

P = 0.203, Fig. 4b), ovary mass (controlled for body mass, LME, P = 0.624, Fig. 4c), egg size (LME, P = 0.927, Fig. 4d) or egg survival over 30 days post fertilization (30 dpf; Binomial GLMM, P = 0.814, Fig. 4e) were not compromised by the pellet diet. Note that the egg number was not different even for a contrast in female diet only (GLMM, P = 0.114). In addition, there was no difference in the egg developmental stages at 30 dpf (Pearson's χ^2 test, P = 0.054, Fig. 4f), suggesting comparable effects of the two diets on embryo development of the next generation.

Discussion

The standardized diet is necessary for full establishment of model organisms[9,10]. The present study compared turquoise killifish key fitness traits between two diets – widely used bloodworms[2] and BioMar INICIO commercial pellets. Overall acceptance of pellets was good from puberty (21 dph) onwards. A minor dietary effect was found on body mass probably via differences in visceral fat storage. Differences in diet-dependent energy storage also occurred at the hepatocellular level. A sex-specific effect of diet was detected in liver mass with heavier livers in bloodworm-fed males but not females. Standard length, Fulton's condition, extent of hepatocellular vacuolation and reproductive parameters did not differ considerably between diets suggesting the suitability of the pelleted diet for *N. furzeri*. The similar performance of bloodworm-fed fish compared to pellets-fed fish was compensated by 7.5-fold higher mass of consumed bloodworms. We believe that this study represents a promising step towards *N. furzeri* husbandry standardization a prerequisite for inter-laboratory comparison between studies.

Growth rate often affects lifespan[28,36] and, at the same time, it is a widely used parameter to compare diet quality[4,37]. Turquoise killifish body size was similar for both diets during the entire experimental period. Body mass (but not body size) was initially slightly higher in pellet-fed fish but the difference disappeared in males upon reaching their asymptotic size (56 dph). Initial higher body mass in the pellet group can be related to the marginally higher proportion of males in that group (Supplementary Table S1), but the sex of juveniles is indistinguishable and the male-bias was only detected after fish reached maturity. Body mass also increased faster in the pellet-fed group than in the bloodworm-fed group between age 15 and 30 days. This can be associated with faster growth of pellet-fed males compared to bloodworm-fed males and, consequently, their earlier maturation related to faster development of sexual characteristics, such as deeper body and development of gonads. In addition,

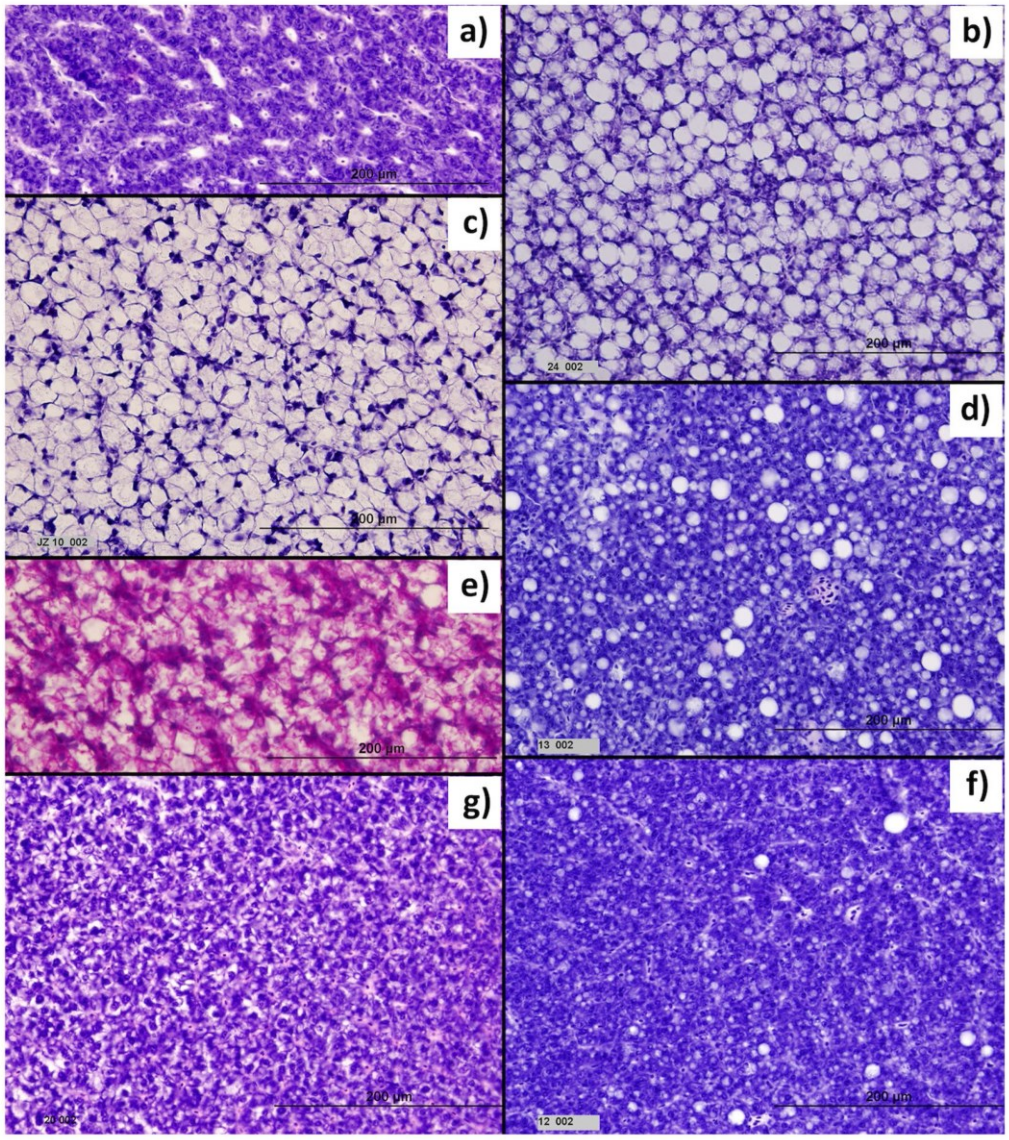


Figure 3. Various types of hepatocellular vacuolation of *Nothobranchius furzeri* and extent of hepatocellular vacuolation stained with Mayer's hematoxylin and eosin (a, b, c, d, f, g) and periodic acid Schiff reaction (e) under 175× magnification. (**a**) Liver parenchyma with no apparent hepatocellular vacuolation, (**b**) macrovesicular lipid type hepatocellular vacuolation, in analysis considered as lipid type vacuolation, (**c**) glycogen-like type severe hepatocellular vacuolation, in analysis considered as glycogen-like type vacuolation, (**d**) mixed size of lipid vacuoles, in analysis considered as lipid type vacuolation, (**e**) section with glycogen-like type vacoulation stained with periodic acid Schiff reaction. Note stained content of vacuoles. (**f**) mostly microvesicular lipid type vacuolation, in analysis considered as lipid type of vacuolation, (**g**) glycogen-like type of minimal hepatocellular vacuolation, in analysis considered as glycogen-like type vacuolation.

the higher fat deposition could have already been manifested at that age. Altogether the results suggest better nutritional status in pellet diet group than bloodworm group, likely related to the higher lipid content of pellets.

Condition is often used as a marker of health status in fish[8]. Fulton's condition factor did not differ between dietary groups, but the visceral fat score in the pellet group was much higher, indicating their superior somatic condition. This inconsistency can be related to considerably bigger livers in bloodworm-fed males which could mask the tendency for lower condition in bloodworm-fed group. All fish were fasted prior to dissection and had empty guts. Hence, differential gut fullness could not have been responsible for the difference in condition factor. High visceral fat loads in fish fed on pellets are common[38,39], but this does not necessarily shorten lifespan[8]. The most probable cause of high visceral fat in pellet-fed fish is that the fat content in pellets is 3-5 times higher than bloodworms (Table 1). The increased level of fat accumulation could potentially become a study model for dietary

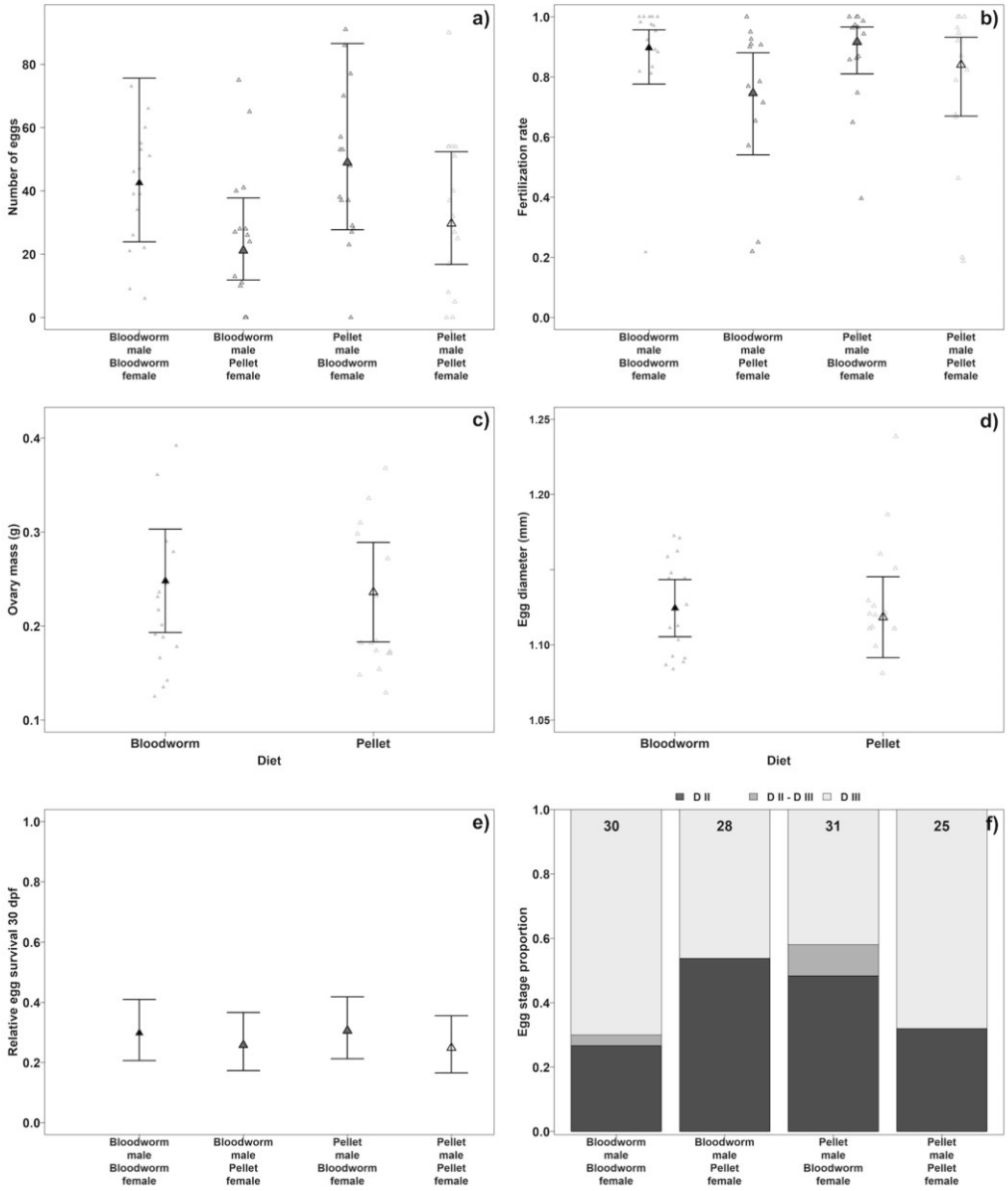


Figure 4. Reproductive parameters in pellet-fed and bloodworm-fed dietary groups. (**a**) Fecundity. Raw data points and Negative-Binomial GLMM estimated means and 95% confidence intervals (CI) of four combinations of pairs. (**b**) Proportion of fertilized eggs from four pair combinations. Means and 95% CI are Binomial GLMM estimated values. (**c**) Reproductive allotment indicated by ovary mass corrected for eviscerated body mass. Model (LME) estimated means and 95% CI and raw observation points. (**d**) Egg diameter. Model (LME) estimated mean and 95% CI. Observation points are female-specific mean egg sizes. (**e**) 30 days post fertilization (dpf) survival of egg individually incubated in water. Mean and 95% CI are Binomial GLMM estimated values. Raw data are not presented due to their binomial nature. (**f**) Egg diapause stage proportion after 30 dpf. Values in the upper part of bars are sample sizes from which diapause stage was determined.

induced obesity[40]. At the same time, high fat accumulation suggests that formulation of a turquoise killifish specific formula for pellets is desirable.

Fish hepatocytes are more vacuolated than mammal hepatocytes[18]. Wild annual killifish have an exceptionally high extent of hepatocellular vacuolation[20,21], suggesting that it may be a natural state. In the present study, male livers were more vacuolated than females' which is in accordance with previous findings both in captivity[22] and the wild[20]. Fish fed pellets possessed almost exclusively lipid type hepatocellular vacuolation which is in accordance with other studies of fish fed on pellets[18,38]. On the other hand, the bloodworm-fed group had a similar extent of hepatocellular vacuolation, though predominantly of the glycogen-like type. Higher liver glycogen reserves develop when fish consume a diet rich in carbohydrates[41] and bloodworms had 2-3 times higher

carbohydrate content than pellets. This differentiation between the two types of energy reserves in livers provides an interesting model for comparative physiology.

The sex-specific reproductive role affects liver size[42]. Overall, males had larger livers than females and female liver size did not differ between dietary groups. We speculate that the lack of difference in female liver size between dietary treatments is related to energetically demanding oocyte production, directly linked to liver metabolism[5]. Higher reproductive energy mobilization in females is also indicated by their lower hepatocellular vacuolation extent and lower visceral fat accumulation. Male gamete production is less energy demanding[43] and allows them to store more energy reserves in their livers. Glycogen storage increases liver size[44] and extensive glycogen reserves were found in bloodworm-fed males, making this the most likely cause of their large livers.

The dominance of glycogen-like vacuolation in hepatocytes of the males kept on bloodworm diet accentuates the need to distinguish between physiological and pathological accumulation of glycogen. The solution lies in the understanding of subcellular changes, which requires information from transmission electron microscopy. Similarly, simultaneous occurrence of PAS positive large vacuoles and nuclei in submembrane position deserves further attention. Our findings seem to contradict the widely accepted histological definitions of glycogen vacuolation in fish by Wolf & Wolf (2005)[18]. For our experimental fish we suggest a two-step cell alteration, i.e., lipid macrovesicular vacuolation followed by glycogen accumulation.

Generally in fish, a pellet diet affects the reproductive parameters of females[13,14], but less so of males[8,45]. The present study partly supports this, because there was no difference in fertilization ability in pellet fed males but a non-significant trend for lower fecundity and fertilization rate in females. Otherwise we did not detect any effect of diet on female reproductive allocation (ovary mass and egg size) indicating that females rather respond to feeding periodicity[46] than to macronutrient composition. There was no difference in the developmental stage at 30 dpf, suggesting comparable effects of two parental diets on the dynamics of embryo development.

Good acceptance and ingestion of a newly introduced diet is necessary for its successful establishment in laboratory studies. In the present study, BioMar INICIO - commercially available formulated diet, was well accepted by killifish from onset of puberty onwards. Commercial pellets are not standardized (purified) diet and their nutrition composition may vary by product batch[10], despite that laboratory analysis of our pellets matched composition provided by the manufacturer. It should be noted, that fish fed by pellets produced fewer feces, reducing the need of frequent water exchange. Also feeding by pellets was less financially demanding and less laborious than feeding by bloodworms.

The challenging transition of juvenile fish to a formulated diet is a common problem in aquaculture[37]. It is possible to feed juvenile killifish pathogen free, but nutritionally unstable, bloodworms during the juvenile transition period to pellets (e.g. Hikari Bio-Pure bloodworms, Japan, http://www.hikariusa.com). However, we have a recent experience that transition from *Artemia* nauplii directly to pellets is possible (J. Žák, personal observation). Yet, in turquoise killifish, it is necessary to train fish for satisfactory pellet acceptance (J. Žák, personal observation). We highlight that the age around the onset of puberty and maintenance in social tanks are optimal conditions for a successful killifish transition to pellets which was previously found also in zebrafish[47]. We do not believe that the short period when killifish were co-fed bloodworms and pellets affected our results, because fish were fed solely on pellets for 35 days during which they gained 4-10 times their initial body mass. However, we acknowledge that the co-feeding transition period could be a time window for disease or chemical contamination from bloodworms[5]. Otherwise, the combined diet of commercial pellets with live food improves the reproductive parameters of fish[14,48] and can be recommended as a strategy for fecundity increase until the reproduction-enhancing diet will be developed (such as in broodstock commercial fish[49]).

The present study demonstrates that turquoise killifish can be kept on a diet of dry pellets, potentially enabling research into the development of standardized (purified) diets which would enable to study effect of macronutrient manipulation on life histories[8]. We believe that our findings are applicable to most medium to large-sized *Nothobranchius* species; we were also able to convert adults of *N. orthonotus*, *N. kadleci, N. melanospilus* and *N. guentheri* to pellets (J. Žák, personal observation). Future studies should determine whether pelleted diet influences turquoise killifish lifespan, should develop the protocol for feeding formulated diet to early ontogenetic stages and should specify nutritional requirements of *N. furzeri*. We believe that the optimal formulated diet should contain a lower proportion of fat than that contained in the pellets used in the present study and a lower proportion of carbohydrates than in bloodworms. Ultimately, open formula of purified (chemically defined) standardized diet must be developed to reduce diet-associated variability in the organismal responses[10]. We believe that present results provide first important step towards future development of the standardized laboratory diet for *N. furzeri*.

Methods

All methods and procedures were carried out in accordance with relevant guidelines and regulations of the Czech Republic. Experimental facility and handling protocols were approved according to national laws No. 246/1992 and No. 419/2002 and by Ministry of Agriculture (breeding facility No. CZ 62760203, permit approval document 62116/2017-MZE-17214 dated 20 October 2017).

Killifish origin and housing. All experimental work was completed on the wild-derived strain MZCS 222[50] of turquoise killifish (*Nothobranchius furzeri*). Detailed fish husbandry, water quality and size assortment is described in Supplementary material (Supplementary section - Killifish housing, Water quality, Supplementary Table S1, S2). In short, fish were hatched and raised in the common tanks until 12 days post hatching (dph) following Polačik *et al.* (2016)[2]. Thereafter they were moved to 35 L tanks with three replicates (cca 30 fish per tank) per dietary treatment and size sorted at 15, 17 and 20 dph (Supplementary Table S1) from the initial density of 30 fish per tank (n = 180 fish, 3 tanks per treatment) to 10 – 21 fish per tank (5 tanks per treatment). Size assortment was done to improve the growth and to reduce aggression[2]. Keeping fish in social groups improves

fish willingness to feed and therefore promotes easier recognition of new food items[51]. At the age of 29 dph, the final experimental groups (four replicates per treatment, 4 males + 8 females per 35 L tank) were established. The experiment was terminated when fish reached asymptotic growth at the age of 56 dph. Water temperature was kept at 27.1 °C ± 0.87 (mean ± SD) and light regimen was 14 L:10D.

Killifish feeding procedure. After hatching, the fish were fed live *Artemia* nauplii (Sanders, USA, www.gsla.us) three times per day (8:30, 13:15, 18:15) in order to provide continuous access to live *Artemia*. Finely chopped bloodworms (Chironomidae, Petr Grýgera, Czech Republic, https://nakrmryby.cz/) started to be added to the diet at the age of 12 dph. From this point onward, the control group was fed exclusively with bloodworms but in the experimental group we started to supplement the bloodworms with the smallest available grade of dry food pellets BioMar INICIO 0.4 mm (Denmark, https://www.biomar.com/). At the age of 15 dph, several fish were observed to accept pellets. Pellets were soaked in water for 1-3 min to soften them before being added to the tank in batches of 1 to 5 pellets using a Pasteur pipette. Fish were fed *ad libitum* (amount consumed within 5 min, Supplementary Fig. S1) with bloodworms or pellets three times per day. The pellet size was gradually increased in 0.2 mm steps (from 0.4 mm to 1 mm and 1.5 mm; Supplementary Fig. S3), larger pellets being offered prior to the smaller ones until they were fully adapted to the larger size (usually 2-3 days). Detailed information on pellet size with respect to the experimental stage can be found in Supplementary Fig. S3. The mixed diet (bloodworms and pellets) continued up to the age of 21 days, when all experimental fish fully accepted the pellets. At this point the schedule was reduced to twice a day (11:30, 18:45). The age of 21 days coincided with the onset of coloring up in males. From this point onwards, experimental fish received exclusively pellets until the termination of the experiment. Dry pellets were fed from the age of 38 days and were vigorously accepted.

Prior to each feeding, food mass was determined. Before feeding, thawed bloodworms were left for 5 minutes in the sieve to dry and then weighed to the nearest 0.001 g using an analytical scale (Kern PCB 350-3, www.kern-sohn.com, Germany). The same procedure was done with unused bloodworm after feeding. The difference in mass before and after feeding was taken as the amount of bloodworms consumed. A smaller dose of pellets than the fish were expected to consume was weighed to the nearest 0.001 g in small plastic cup prior each feeding. Then small weighted amounts of pellets were given to fish until they were fully satiated.

The food conversion ratio (FCR = food intake / body mass gain) was computed for each diet at each body mass sampling point. The macronutrient content (Table 1) of bloodworms (and BioMar INICIO 0.8 mm as a control for method calibration), were analyzed in an accredited laboratory at the National Veterinary Institute in Olomouc, Czech Republic (https://www.svuolomouc.cz/).

Somatic parameters. We measured body size (SL, standard length: excluding caudal fin, mm), body mass (BM, g), specific growth rate (SGR, % × day^{-1}), Fulton's condition factor (K; body mass divided by cubic SL and multiplied by 100), visceral fat score (VFS, 0-3), liver mass (LM, g), hepatocellular vacuolation extent (HVE) and type of hepatocellular vacuolation (THCV) were determined. SL and BM for all experimental fish were measured every five days between age 15 and 30 dph and every seven days from 30 dph to 51 dph for all experimental males and a random subsample of 32 females (4 per each aquarium). The last measurement was completed at the age of 56 dph. BM was measured on towel-dried live specimens to the nearest 0.001 g using analytical scales. SL was measured using ImageJ v1.46 (NIH, Bethesda, MD, USA) from photographs in a plastic container with shallow water and scale. SGR was computed from sampling-point-pooled averages for both SL and BM with the formula: $SGR = (\ln S_2 - \ln S_1) \times (100/t)$ where S_2 is average terminal size (SL, mm or BM, g), S_1 is initial average size and t is the length of time interval in days. Condition K[52] was calculated at the end of the experiment. Fat score – VFS was visually determined after opening the body cavity at the end of the experiment (scale 0–3; 0 – no visceral fat; 3 – internal organs completely covered by fat). Liver mass - LM of freshly extracted livers was weighted to nearest 0.001 g using analytical scales.

Liver histology. The liver was removed from the body cavity, fixed in Davidson's fixative (for 48 h), stored in 70% ethanol (4 days) and processed using the paraffin technique. This included dehydration in series of ethanols, aceton and xylen, and embedding in Histoplast (Serva, Germany, https://www.serva.de/enDE). To ensure the best possible prerequisites for comparison, liver samples taken from 41 individuals (10-11 per treatment-sex combination, 2-3 per sex-tank combination) were oriented identically while embedded into Histoplast blocks. From the parietal part of the liver, five semi-serial 4 µm sections per fish were prepared using a rotating microtome HM360. Of these, three sections per fish were stained with Mayer's hematoxylin and eosin. The histological findings were documented using a BX60 Olympus microscope equipped with a DP71 camera under 175× magnification. The images of representative sections were analysed for HVE in ImageJ that calculated the proportion of total unstained area (mainly vacuoles) against the well-stained tissue. This method was validated by re-examining a subsample of 24 sections by another experienced evaluator and there was a strong association between both results (Pearson's correlation coefficient, $r = 0.76$, $t_{22} = 5.51$, $P < 0.001$). THCV was evaluated blindly, based on vacuole character; glycogen-like type vacuolation (Fig. 3c,e,g: irregular vacuoles with indistinct margins[19]; lipid type vacuolation (Fig. 3b,d,f: a clear round vacuoles with sharp edges)[19]. Several sections with liver tissue containing irregular vacuoles in the cytoplasm of hepatocytes (glycogen-like THCV) were validated by supplementary staining for glycogen (periodic acid Schiff reaction, Fig. 3e).

Reproductive parameters. To compare fecundity and fertilization rate between treatments, 16 males (from a total of four tanks) and 16 females (from a total of two tanks) per treatment were spawned in a 2 L plastic container with substrate of 0.5 cm fine grained sand for 2 hours[2]. Experimental spawning took place at the age of 53 and 54 dph respectively. Each female was spawned twice – in a random order with a male from the same treatment group and with a male from the other treatment group. This design was selected to address potentially confounding parental effect on reproductive parameters. Fish were spawned in this setting twice before (age 51

Compared parameter (statistical method)	Model	Sample size per dietary group or combination	Sex-specific sample size per group	Sample size per tank	Total sample size
SL 15 days (LME)	SL15 ~ diet + (1|tank)	90	90 J	30 J	180
SL 30 days (LME)	SL30 ~ diet + (1|tank)	32	16 M:16 F	4 M:4 F	64
SL 56 days (LME)	SL56 ~ diet + (1|tank)	32	16 M:16 F	4 M:4 F	64
BM 15 days (LME)	BM15 ~ diet + (1|tank)	90	90 J	30 J	180
BM 30 days (LME)	BM30 ~ diet + (1|tank)	32	16 M:16 F	4 M:4 F	64
BM 56 days (LME)	BM56 ~ diet + (1|tank)	32	16 M:16 F	4 M:4 F	64
SGR (Gaussian LM)	SGR ~ diet + sampling point	7	—	—	14
Fulton's condition (LME)	K ~ diet + sex + (1|tank)	32	16 M:16 F	4 M:4 F	64
VFS (MPA)	VFS ~ diet + error (tank)	32	16 M:16 F	4 M:4 F	64
LM (LME)	LM ~ diet + BMdis + (1|tank)	32	16 M:16 F	4 M:4 F	64
Additional LM (LME)	LM ~ diet + HVE + BMdis + (1|tank)	19-21	10 M:10 F	2-3 M:2-3 F	40
HVE (LME)	HVE ~ diet + sex (1 | tank)	20-21	10 M:10-11 F	2-3 M:2-3 F	41
THCV	Pearson's χ^2 test	19-21	10 M:10 F	2-3 M:2-3 F	40
N of eggs (Neg.Bin. GLMM)	N eggs ~ pair combination + BM + (1|tank)	16	16 M:16 F	4 M:8 F	32/2358
FR (Bin. GLMM)	FR ~ pair combination + (1| TM.TF)	16	16 M:16 F	4 M:8 F	32/2358
Ovary mass (LME)	OM ~ diet + BMdis + (1|tank)	16	16 F	4 F	32
Egg diameter (LME)	ED ~ diet + (1|tank/ID.female)	16	16 F	4 F	32/837
30dpf egg survival (Bin. GLMM)	ES ~ diet + (1|dish)	100	—	—	400
30 dpf egg diapause stage	Pearson's χ^2 test	25-31	—	—	114

Table 2. Overview of statistical models with sample sizes. TM.TF is combined factor made by joining tank identity of male and tank identity of female. The number behind the slash symbol is total number of eggs analysed. Models with missing sex factor were performed separately for each sex due to significant sex-specific interaction (or only completed for females in the case of reproductive parameters). LME – Linear mixed effect model[56]; GLMM – Generalized linear mixed effect model[56]. In all binomial models raw data were used instead of percentages to account for sample size. MPA – Multistratum permutation analysis with specified 10000 iterations[57]. χ^2 analyses were completed by Pearson's chi-squared test with simulated p-value (based on 10000 replicates). J: juvenile; M: male; F: female. For parameter coding refer to "Experimental procedures".

and 52 days respectively) for habituation and standardization. After experimental spawning, each female was weighed to 0.001 g and males and females were released into separate tanks to prevent spawning.

From each pair combination, survival of 100 individually incubated eggs (4 plastic dishes with 25 compartments) was determined. The maximum egg contribution from each female in a respective day to the egg pool from which 100 eggs were selected was 20. Eggs were incubated in 25 °C water in a laboratory incubator (Q-Cell, Pol- lab, www.poll.pl). After 30 dpf, the developmental stage of the surviving eggs was determined[53]. Reproductive allotment was estimated from ovary mass[46] at the age of 56 dph. Ovaries were extracted from the body cavity and fixed in 4% formalin for later measurement and egg extraction for egg size measurement[46]. Egg size was measured for a batch of 10 – 51 eggs per female.

Statistical analysis. All statistical analysis was carried out in R environment 3.5.2[54]. All possible two way interactions were included in models but removed when non-significant. In case of a significant interaction with sex (SL30; SL56; BM30; BM56; LM, VFS), separate sex-specific analyses were performed. For statistical analysis of SL30; SL56; BM30; BM56; LM, VFS and ovary mass, 4 females per tank were randomly selected to retain a balanced dataset. Statistical difference of K was interpreted only between dietary groups but not between sexes, as the sex difference is not informative in sexually dimorphic species[55]. In THCV statistical analysis, a single individual with no hepatocyte vacuolation was omitted due to its rarity. Additional analysis revealing the effect of HVE on liver size was completed with THCV as a fixed factor (instead of treatment as the fixed factor) due to their strong collinearity (Fig. 2d). The proportion of individuals with pre-neoplastic lesions was too few to analyze (4 individuals out of 41 examined). A detailed overview of sample sizes and statistical models can be found in Table 2. Complete tables of statistical results can be found in the supplementary material (Supplementary Tables S3-S30).

Data availability

Original data supporting the findings of this study are available via Figshare repository (https://doi.org/10.6084/m9.figshare.9825068)

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Author contributions

J.Ž. designed the study, I.D. did the histological analysis, J.Ž. and I.D. collected the data, J.Ž. analysed the data, J.Ž., M.R. and I.D. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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PAPER 11: *Nothobranchius* histopathology

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RESEARCH ARTICLE

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Histopathology of laboratory-reared *Nothobranchius* fishes: Mycobacterial infections versus neoplastic lesions

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Abstract

Short-lived killifishes of the genus *Nothobranchius* Peters, 1868 (Cyprinodontiformes) are considered promising model organisms for biomedical research on ageing and tumorigenesis. We conducted histopathological analysis of 411 adult individuals from three *Nothobranchius* species to study details on spontaneous age-related neoplastic lesions. Light microscopy based on H&E and toluidine blue-stained sections revealed (a) non-proliferative liver changes with pronounced vacuolation of hepatocytes; (b) proliferation of kidney haemopoietic tissue contributing to excretory system damage; (c) proliferation of splenic mononuclear haemoblasts accompanied by reduced erythropoiesis; (d) proliferation of mononuclear cell aggregates in the liver parenchyma; and (e) rare occurrence of hepatocellular adenomas. Ziehl–Neelsen (ZN) staining revealed that the proliferative lesions are a host defence response to mycobacterial infections manifested by activation of the mononuclear phagocytic system and atypical granulomatous inflammatory reaction. 16S rRNA analysis identified three species of *Mycobacterium* in our samples. Our findings turn attention to lesions which mimic neoplasms by their gross appearance and question the light microscopic interpretation of lesions unless differential ZN staining is included. Beyond the limitations of our morphological approach, the intensity of mycobacterial infections is a challenging opportunity for research into the molecular-genetic background of the mononuclear phagocytic system reaction in *Nothobranchius* killifish.

KEYWORDS

mononuclear phagocytic system, mycobacterial infections, proliferative lesions, tumour misdiagnosis

1 | INTRODUCTION

Teleost fishes with short lifespans have a special position among the fish species which are used in laboratory research. They were introduced as model organisms for ageing studies by Markofsky (1979), Markofsky and Milstoc (1979) and Cooper et al. (1983). Since then, the importance of annual killifish, especially *Nothobranchius furzeri* (the shortest-lived vertebrate currently cultured in captivity), for ageing studies has been highlighted by a series of key articles (Baumgart et al., 2015; Blažek et al., 2017; Cellerino et al., 2016; Di Cicco et al., 2011; Harel et al., 2015; Hu & Brunet, 2018; Terzibasi et al., 2007; Valdesalici & Cellerino, 2003; Wendler et al., 2015).

Studies on spontaneous lesions and the cause of death form an important part of the research on short-lived fish and annual killifish in particular. Di Cicco et al. (2011) performed an analysis of spontaneous lesions in organs of *N. furzeri* and found histopathological changes in the liver (90% of individuals), kidney (75%), heart (70%) and gonads (40%). A high proportion of these changes were considered to be neoplasias classified by these authors as hepatic and

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renal carcinomas, respectively. The diagnosis was supported by immunohistochemical and genetic markers but morphological features were not well described, being referred to as nodules of neoplastic tissue and documented in low magnification images of organ sections. The same images (Di Cicco et al., 2011) were reproduced in a key review by Cellerino et al. (2016) who, in accordance with Di Cicco et al. (2011), presented *Nothobranchius* fishes as organisms particularly suited for the investigation of biological and molecular aspects of ageing and ageing-associated dysfunctions. An identical classification of lesions in the livers of *Nothobranchius furzeri* and *N. korthausae* was published by Baumgart et al. (2015) who also stressed the age-related onset of hepatomas and hepatocarcinomas observed in the same organ sample. Similar opinions on liver tumours and tumour-associated kidney swellings were incorporated into a study on the intraspecific divergence in life span and ageing of African annual fishes by Blažek et al. (2017).

A different opinion on the nature of histopathological changes in the kidney and liver of ageing *N. guentheri* was published in an earlier, well-documented paper for its time, by Cooper et al. (1983). These authors confirmed previous observations by Markofsky (1979) and Markofsky and Milstoc (1979) and classified histologically similar cell aggregates found in the kidney and liver as a nodular type of histiocytic lymphoma.

Studying these problems, one should not ignore the potential role of mycobacteria, which are ubiquitously distributed microorganisms and a known problem also in zebrafish research facilities and cyprinodont fish used for toxicological and carcinogenicity assays (Kent et al., 2004; Watral & Kent, 2007; Whipps et al., 2012). In terms of our objectives, one of the most relevant studies is that by Broussard et al. (2009) who experimentally tested cancer risk associated with chronic *Mycobacterium marinum* infections in Japanese medaka *Oryzias latipes* (Temminck & Schlegel, 1846). Results of these experiments did not prove a direct correlation; however, it revealed mycobacteria in liver lesions similar to those classified in the above-mentioned *Nothobranchius* studies as hepatic carcinomas.

Despite intensive exploitation of *Nothobranchius* fishes as emerging model organisms for research on ageing (Hu & Brunet, 2018; Platzer & Englert, 2016), significant gaps in the knowledge of cellular and tissue manifestations of *Nothobranchius* disease conditions are apparent. In order to supplement such data, we studied spontaneous histopathological lesions in laboratory-reared *Nothobranchius* fishes with the aim of obtaining a general overview of spontaneous and proliferative lesions specifically and compiling a histopathological classification of these changes.

2 | MATERIAL AND METHODS

2.1 | Fish

The principal research subjects of the study were two strains (MZM 0410 studied in 2019 trial and GRZ studied in 2020 trial) of the turquoise killifish *Nothobranchius furzeri* ($N = 247$) bred according to the protocol by Polačik et al. (2016). Adult fish were fed once daily ad libitum. A pelleted diet (Biomar Inicio) was fed to one-third of the fish in the GRZ trial, one-third fed Grygera bloodworms and the remaining third Petra-Aqua bloodworms. The MZM trial was fed Grygera bloodworms. The diet did not have a significant effect on our findings (Table S1) and thus was not considered in our final conclusions. Comparative material ($N = 80$) was obtained from *Nothobranchius* fishes bred in 2013, the housing details of which are presented in Blažek et al. (2017). Complementary material contained *Nothobranchius* fishes ($N = 84$) examined in diagnostic services rendered by us to local fish breeders (housing not known) in 2000–2010 (Table 1).

2.2 | Material examined

Histopathological examination included samples of the kidney, liver, spleen and, if indicated, swim bladder of *Nothobranchius furzeri* MZM 0410 strain (MZM hereafter) collected in six samplings from individuals aged 6–21 weeks ($N = 78$) and of *N. furzeri* GRZ strain sampled at the terminal age of 12–19 weeks ($N = 169$). This recently collected material from 247 individuals was supplemented by re-examination of previously sectioned material on organ lesions of a total 411 *Nothobranchius* fishes (Table 1).

2.3 | Sample collections

The sampling methods for histological examinations applied in MZM and GRZ strains consisted of euthanasia with an overdose of clove oil, fast, careful and sterile removal of the left-side body wall followed by organ size scoring of the kidney, liver and spleen of each individual. Kidney size was scored on 5-point ordinal scale (0—normal size, 1—mild, 2—moderate, 3—severe and 4—extreme kidney enlargement). Liver and spleen size was scored binomially (natural size/enlarged organ). Colour changes of liver and spleen were recorded binomially as natural or altered colour. After organ scoring and extraction, organs were divided into two parts, one of which was frozen in dry ice and stored for future genetic studies (not part of this study) while the other was fixed for histopathological study.

2.4 | Sample preparation for histology

MZM organ samples, divided into subsamples of suitable size, were fixed in 2% osmium tetroxide, dehydrated in acetone dilution series and embedded in Spurr resin for examination of semithin and ultrathin sections using light or electron microscope, respectively. GRZ organ samples were fixed in 10% neutral-buffered formalin (NBF) and processed using the standard paraffin technique. The same technique was applied to samples from 2013 and archived material from diagnostic history of *N. furzeri*, *N. kadleci* and *N. orthonotus* (see Table 1) fixed in NBF or Davidson fixative. All paraffin

TABLE 1 Sectioned material of *Nothobranchius* fishes examined in the study

Fish species	Strain	No. of fish examined	Organs examined	Material type examined
N. furzeri	GRZ	169	L, K, S, SB	Paraffin sections (2020)
	MZM 0410	78	L, K, (S)	Semi- and ultrathin sections (2019)
	NF 121	20	L	Paraffin sections of 2013 study[a]
	NF 222	20	L	Paraffin sections of 2013 study[a]
	unknown	69	EF incl. SB	Paraffin sections (diagnostic history[b])
N. kadleci	NK91	20	L	Paraffin sections of 2013 study[a]
	NK430	20	L	Paraffin sections of 2013 study[a]
	unknown	3	EF incl. SB	Paraffin sections (diagnostic history[b])
N. orthonotus	unknown	12	EF incl. SB	Paraffin sections (diagnostic history[b])
Total		411		

Abbreviations: EF, (entire fish); K, (kidney); L, (liver); S, (spleen, incomplete set of samples); SB, (swim bladder).
[a]Re-examined sections (material not available for re-sectioning).
[b]Diagnostic history (individual cases examined from 2000 to 2010).

sections evaluated in the study were stained with haematoxylin and eosin (H&E). Ziehl–Neelsen (ZN) differential staining (Roberts, 1978) of parallel/semiserial sections was used for identification of acid-fast organisms such as members of the genus *Mycobacterium*. Bacterial presence in MZM was noted when rod-shaped bacteria were detected in ultrathin sections of MZM during slide examination.

2.5 | Histological evaluation

The evaluation of histopathological changes was based on the generally accepted diagnostic criteria for degenerative, inflammatory, proliferative non-neoplastic and neoplastic lesions using the standardized nomenclature for proliferative and non-proliferative lesions in fish (Boorman et al., 1997; Goodman, 2007; Keenan et al., 2015; Spitsbergen et al., 2012; Thoolen et al., 2010; Wolf et al., 2015) and the specific criteria for distinguishing hyperplastic from neoplastic lesions (Fournie et al., 2005). Our stepwise diagnostic process followed the recommendation of Orazi (2007) and consisted of screening for the presence ("yes/no") of proliferative lesions, decision on the proliferation type (neoplastic/non-neoplastic), detection of the reactive condition if present and classification of neoplastic proliferation using comparative data available in advanced studies of model tetrapod vertebrates, rats and mice (Thoolen et al., 2010).

2.6 | Species determination of mycobacteria

A partial determination was undertaken of *Mycobacterium* species in kidney tissue samples of 11 *N. furzeri* (4 GRZ and 7 MZM). For full-length 16S rRNA analysis, bacterial DNA was extracted from frozen tissues using ZymoBIOMICS DNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions. 16S metagenomic libraries were prepared using LoopSeq™ 16S Microbiome SSC 24-Plex Kit (Loop Genomics) and sequenced on NextSeq (v2.5 reagents, 300 cycles) to finally generate long-read sequences (~10,000 molecules per sample). Raw reads were processed following the standard Loop Genomics protocol for full-length 16S sequencing. Sequence results were processed through the SILVA138.1 Small Subunit rRNA Database and Global Catalogue of Microorganisms (Type Strains Genome Database). 16S rRNA hypervariable regions V3/V4 were analysed according to Klindworth et al. (2013).

For whole-genome metagenomic analysis, DNA was extracted from frozen tissues using DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. Libraries were prepared using Truseq DNA PCR-free Low Throughput Library Prep Kit (Illumina) and sequenced using Illumina NovaSeq 6000 System (NovaSeq 6000 S2 Reagent Kit, 200 cycles). Raw sequencing data were mapped to the NFU reference NFZv2.0 (Bradshaw & Valenzano, 2020) using the BWA tool (Li, 2013). Unmapped reads were extracted using samtools (Li et al., 2009). An accurate strain-level microbial profiling was performed using the MetaPhlAn tool (Beghini et al., 2020) on extracted reads with the latest database of unique clade-specific marker genes (i.e., mpa_v30_CHOCOPhlAn_201901). Based on the results, a new alignment was set using BWA on unmapped reads to the particular genomes.

2.7 | Statistical analysis

Statistical comparisons of gross necropsy findings and histological findings were done for cohorts of MZM and GRZ, for which appropriate sample sizes were available. In each case, backward selection of the most parsimonious model was performed based on removal of non-significant variables from the model. Overviews of full models, the most parsimonious model and their Akaike information criteria (AIC) are provided in Table S1.

Age dependency of kidney size in MZM was analysed by ordinal generalized additive model (GAM) with kidney size as the ordinal response variable (score 0–4, numbered by one to avoid zeroes)

and sex (male/female) and health status (healthy/moribund) as parametric explanatory variables and age (continuous, $k = 4$) as a semi-parametric explanatory variable. Kidney tissue proliferation in the pooled sample of GRZ and MZM trials and age-dependent kidney tissue proliferation in the MZM trial were analysed by ordinal GAM with kidney tissue proliferation score as the response variable. Liver tissue proliferative changes were analysed by binomial generalized linear model (GLM) only for the GRZ trial, where a reasonable number of proliferative changes was detected. Proliferative change was the response variable (coded as absent/present; macrophage proliferation, mononuclear cell aggregates and necrosis were pooled together as "present"). The details of all full and most parsimonious models are provided in Table S1.

2.8 | Ethics

All methods and procedures were carried out in accordance with relevant guidelines and regulations of the Czech Republic. Experimental facility and handling protocols were approved according to national laws no. 246/1992 and no. 419/2012 and by Ministry of Agriculture (breeding facility no. CZ 62760203, permit approval document 62116/2017-MZE-17214 dated 20 October 2017). The study species are not endangered or legally protected in the wild. All examinations were completed on captive individuals.

3 | RESULTS

3.1 | Gross necropsy findings

Gross lesions observed in *Nothobranchius* individuals of MZM and GRZ strains included changes in the size, shape and colour of the liver, kidney, spleen and swim bladder. Hepatomegaly was regularly associated with a strikingly light/yellow colour of the parenchyma. Renomegaly was frequently found in old MZM adults from the age of 12 weeks, especially in moribund fish (Figure 1a, Table S2. The regular wheat-grain form of the normal kidney was frequently distorted into an asymmetric form with prominent light bulges. Splenomegaly and abnormalities of the swim bladder were found less frequently. Swim bladder abnormalities manifested as a thickening and whitening of the wall, a filling-up of the lumen with a semiliquid material and an enlargement of the gas gland. Detailed histopathological investigations on the swim bladder may be found in Dyková et al. (2020) and Dyková et al. (2021).

3.2 | Histopathological findings

The results of histopathological examinations are summarized for corresponding organs of all study sets despite slight differences in methodology, because the changes in corresponding organs coincided in type. In fact, the study of the MZM cohort based on light microscopy of semithin and electron microscopy of ultrathin sections, the results of which were obtained first, instigated questions about the aetiology of lesions and determined the choice of method for the GRZ study and re-examination of archived material.

3.2.1 | Kidney

Light microscopy of H&E and toluidine blue-stained sections (available from MZM cohort and archived material) revealed proliferation of haemopoietic tissue in the small cranial part of the kidney (corresponding to the head kidney of other bony fish) that was manifested as a monomorphic population of cells with markedly basophilic nuclei (Figures S1–S3). The same type of tissue proliferation was observed in the excretory region of the kidney, together with proliferation of less densely stained mononuclear cells strikingly reminiscent of macrophages (Figure S2a,b). The proliferating cell populations caused an atrophy of glomeruli, displacement of renal tubules and a substantial reduction of their number (Figure S1b–d, S2a). In the most advanced stages of this pathological process, the remnants of altered renal corpuscles were surrounded by a narrow zone of more basophilic mononuclear cells and an abundant population of mononuclear cells resembling macrophages (Figure S2b). They predominated in the foci of bacterial infections seen clearly in the toluidine blue-stained sections. In total, bacterial infections were found in semithin sections of 26% of asymptomatic and 57% of moribund MZM fish, respectively. The intensity of interstitial kidney tissue proliferation reflected quite accurately the semi-quantitative scale of the grossly visible enlargement of this organ in a different manner for kidneys with bacterial presence (Ordinal GAM, edf = 2.871, $\chi^2 = 112.49$, $p < .001$, Figure 1b, Table S3 or without (edf = 2.321, $\chi^2 = 48.96$, $p < .001$). The extent of proliferative lesions was not a function of age in MZM individuals (ordinal GAM, edf = 1.00, $\chi^2 = 1.09$, $p = .296$, Table S4); however, the findings within a specific age group were not always uniform, suggesting variability in individual reactions.

The parallel H&E and ZN stainings of semiserial sections confirmed the association of proliferative lesions with mycobacterial infections in GRZ (ordinal GAM, estimate [SE] = 1.17 [0.43], z = 2.70, $p = .007$, Table S5, Figure 2a,b), and females were more affected by kidney proliferation (1.00 [0.33], z = 3.05, $p = .002$). The intensity of mycobacterial infections (visually estimated density of bacteria in histological sections) varied from quantities in the field of view only just countable to innumerable mycobacteria forming densely packed masses (Figure S3a–c). Maximum densities of mycobacteria were observed in severely proliferated interstitial kidney tissue with predominating macrophages (Figure 2a,b, Figure S3). The latter represented an intense host defence reaction and simultaneously caused the displacement and effacement of renal tubules. The areas of interstitial tissue proliferation were not sharply delimited; they consisted of mononuclear cells with densely stained nuclei (supposed precursors of macrophages) closely surrounding renal tubules, mononuclear

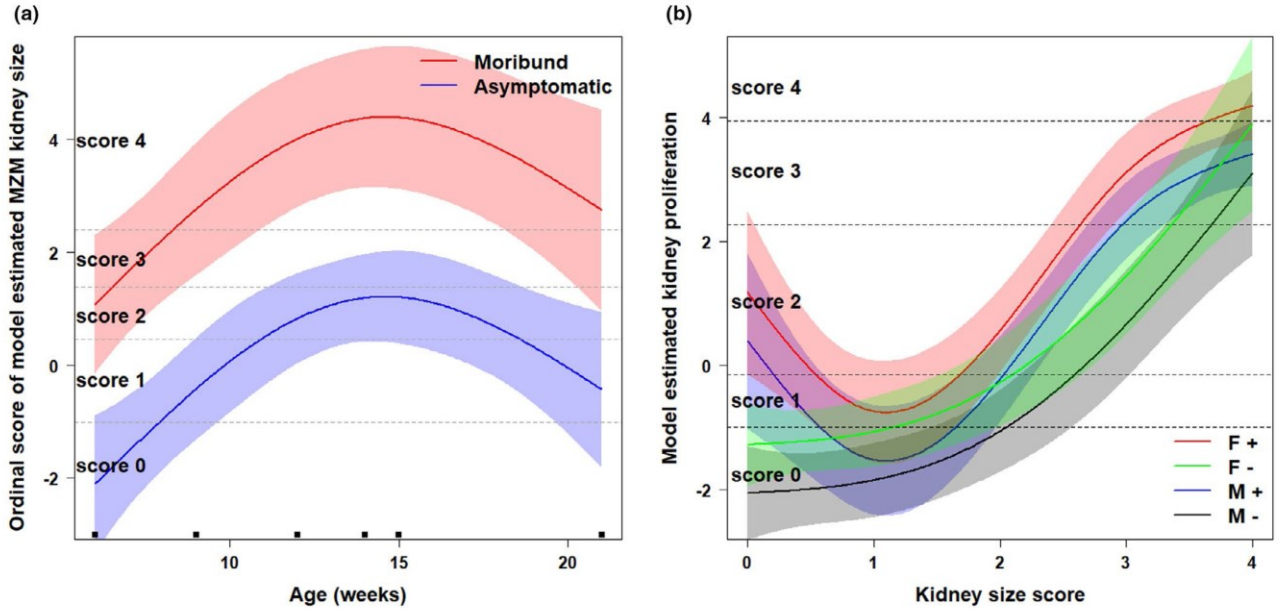


FIGURE 1 Statistical evaluation (ordinal generalized additive model) of kidney size and kidney tissue proliferation. (a) Age dependency of kidney size score in MZM 0410 *Nothobranchius furzeri* cohort. Black squares at bottom represent sampling points. (b) Association of extent of kidney tissue proliferation with kidney size is dependent on sex (F—female, M—male) and bacterium presence (+ positive, – negative) in kidney tissue

macrophages with mycobacteria in the cytoplasm and cell debris intermixed with extracellularly localized mycobacteria. In the course of infection, a misbalance between intensely replicated bacteria and their phagocytic killing was observed. At moderate-to-advanced stages of infection, the host reaction dominated by macrophages was supplemented with atypical granulomatous inflammatory foci. They were characterized by a pronounced alterative/necrotic core and a poorly organized periphery with a minimum walling-off and absence of epithelioid cells. Severe lesions were also observed in the nephrons. Mycobacteria were found to accumulate in dilated Bowman's spaces of the renal corpuscles (Figure S3d,e) and also formed dense clusters/casts in the renal tubules (Figure S3f). Similar findings in the collecting ducts implied an important means of spreading infection.

3.2.2 | Spleen

Spontaneous lesions grossly manifested as splenomegaly and colour changes were found only in adult fish, after the age of 9 weeks (Figures S4,S7). Compared with age-matched apparently healthy fish of three *Nothobranchius* spp. (Figure S4a), the inner architecture of the spleen in infected MZM individuals was significantly changed in that the red pulp was substantially reduced while the white pulp proliferated diffusely (Figure S4b). The distinction between the subcapsular layer of the red versus the white pulp was not clear due to the replacement of erythrocytes in sinusoids by a relatively uniform population of mononuclear cells (Figure S4c). Advanced necrotic changes, together with numerous macrophages containing remnants of senescent cells removed from circulation or engulfed bacteria, were frequently observed in central areas of the organ (Figure S4d). As in the kidney, histopathological lesions in the spleen indicated a severe impairment of its haemopoietic function. The mycobacterial aetiology of the lesions inferred from electron microscopical examination of MZM (Figure S7d) and several extra GRZ samples was confirmed by positive ZN staining in all paraffin sections of the GRZ set. Mycobacteria were responsible for destruction of splenic architecture manifested by an almost complete disappearance of the red pulp, loss of vascular integrity, alteration of ellipsoids that trapped bacteria and domination of large atypical granulomatous lesions characterized by a conspicuous necrotic core with a minimum host tissue reaction rim on their periphery.

3.2.3 | Liver

The examination of the liver parenchyma revealed a series of non-proliferative and proliferative changes, both types usually including disappearance of the polygonal configuration of hepatocytes and loss of their cord arrangement (Figures S5–S8). The most commonly observed (85% of GRZ and MZM trials pooled) non-proliferative lesions were micro- and macro-vesicular vacuolations of hepatocytes consistent with rounded, sharply outlined lipid vacuoles, a glycogen-type vacuolation characterized by large, irregular vacuoles and hydropic degeneration (see figure 3 in Žák et al., 2020). Non-uniform staining of the hepatocyte cytoplasm was rarely seen.

Contrary to expectations, nodular proliferative hepatocellular lesions were found in only 2 of the 411 fish examined (Figure S5a). Light microscopy allowed for identification of these lesions as hepatocellular

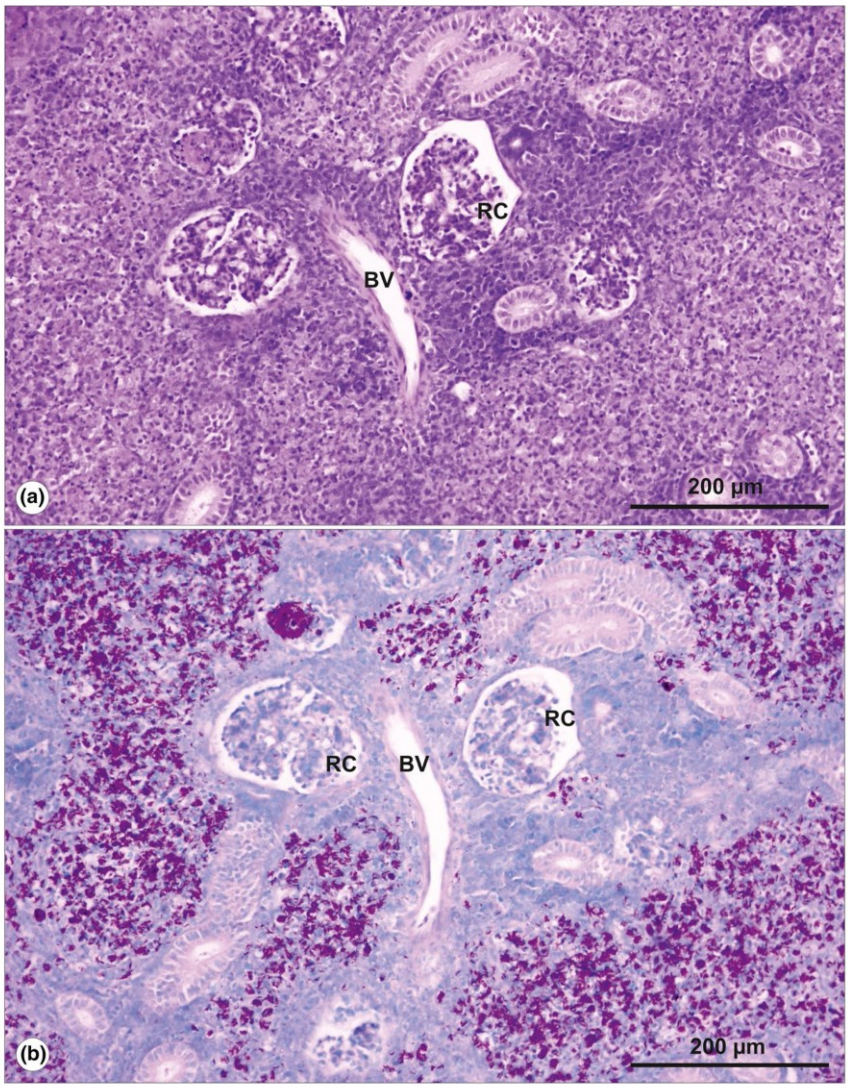


FIGURE 2 Semiserial sections through kidney parenchyma with two renal corpuscles (RC), proliferated interstitial tissue, several convoluted tubules and blood vessel (BV). (a) Section is stained with H&E; parallel section (b) stained with ZN shows dense concentration of mycobacteria in proliferated interstitial tissue rich in macrophages

adenomas or focal nodular hyperplasia (cf., Goodman, 2007). There was a simultaneous mononuclear/monocytic cell proliferation in and among organs of the body cavity in both these cases (Figure S5a,b).

The most frequent (34% from GRZ and MZM together) and typical finding of proliferative changes in the liver parenchyma was a proliferation of cellular components other than hepatocytes (Figure S6,b). In the liver of young individuals, always in close association with blood vessels, solitary structures composed of cells of uniform size were observed, with a delicate capsule and minimum pigment content. These were mononuclear cell aggregates (MAs) morphologically closely related to macrophage centres (MMC sensu Roberts, 1978) alternatively known as pigmented macrophage aggregates (PMA sensu Wolf et al., 2015) or mononuclear phagocyte centres (MPC sensu Wittamer et al., 2011) belonging to the teleost system of innate/nonspecific immunity (Figure S6c). A noticeably increased (albeit not statistically analysed) number of enlarged MAs were observed in older individuals (less frequently in the MZM fish cohort than in NF 222, NF 121 and NK 91). The shape of these MAs was either indistinctly defined or had distinct contours due to the surrounding oedematous changes. A lymphoid cell rim on the MA periphery was found in only one case. In large MAs, there were mild-to-severe oedematous changes leading in advanced stages to the development of multilocular or large unilocular pseudocysts in the liver parenchyma. In some MAs, there were phagocytosed bacteria even if a focus of bacterial infection was not found in the liver (Figure S7a,b). Bacterial infections were confirmed in 26% asymptomatic and 57% moribund MZM fish, respectively. Some cell aggregates containing phagocytosed bacteria were necrotic; however, typical granulomas (a common fish reaction to antigens concentrated by macrophages) were observed only once.

As in the kidney, mycobacterial infection diagnosed in the GRZ cohort was also found to be responsible for proliferative histopathological lesions in the liver (Binomial GLM, estimate [SE] = 2.59 [0.41], z = 6.38, $p < .001$, Table S6). Ziehl–Neelsen staining visualized a transformation of MAs into typical macrophage centres (MC/MPC) in which a high proportion of macrophages characterized by indistinct cytoplasmic borders and abundant cytoplasm with phagocytosed mycobacteria prevailed (Figure 3a–d). Within

these structures, densities of mycobacteria ranged from minimal to moderate, but in general, densities were lower than those in corresponding kidney interstitial tissues (compare Figure 3 and Figure S3). The activation of the mononuclear phagocyte system was manifested in the size of MC and, even more, in their increased abundance. Low numbers of MC characteristic of the initial stages of infection explain lower prevalence of proliferative lesions in the liver (53%; 90 of 169 in GRZ) compared with the kidney (70%). Despite the fact that the changes in the liver and kidney developed in parallel and were dominated by macrophages in both organs, histopathological features differed in the initial stages of infection. They were of diffuse nature in the kidney, whereas in the liver, the less intensive changes were of focal nature, being restricted to MC. In advanced stages dominated by necrotic changes of formerly proliferating cells, lesions in both organs were similar. They represented atypical granulomatous lesions known in infections caused by non-tuberculous mycobacteria.

3.2.4 | Other body cavity organs

Mononuclear cells were also frequently found to proliferate in the body cavity, with devastating impact on the gonads and swim bladder (Figure S9a–c, S10). As in the kidney and liver, the proliferation of monocytes/macrophages was associated with mycobacterial infection. In swim bladders filled almost completely with macrophages containing mycobacteria, proliferation of gas gland epithelium was also observed. In exceptional cases, this proliferation was of neoplastic nature (Figure S11). The connective tissue surrounding an altered swim bladder usually contained a granuloma bearing a striking resemblance to cholesterol granulomas described, for example, in the rat liver (cf. Thoolen et al., 2010, Figure S10a). Among the details which support the systemic character of mycobacterial infections, an incidental finding of severe alteration of the cardiac muscle points to the need for systematic study of the heart (Figure S10b).

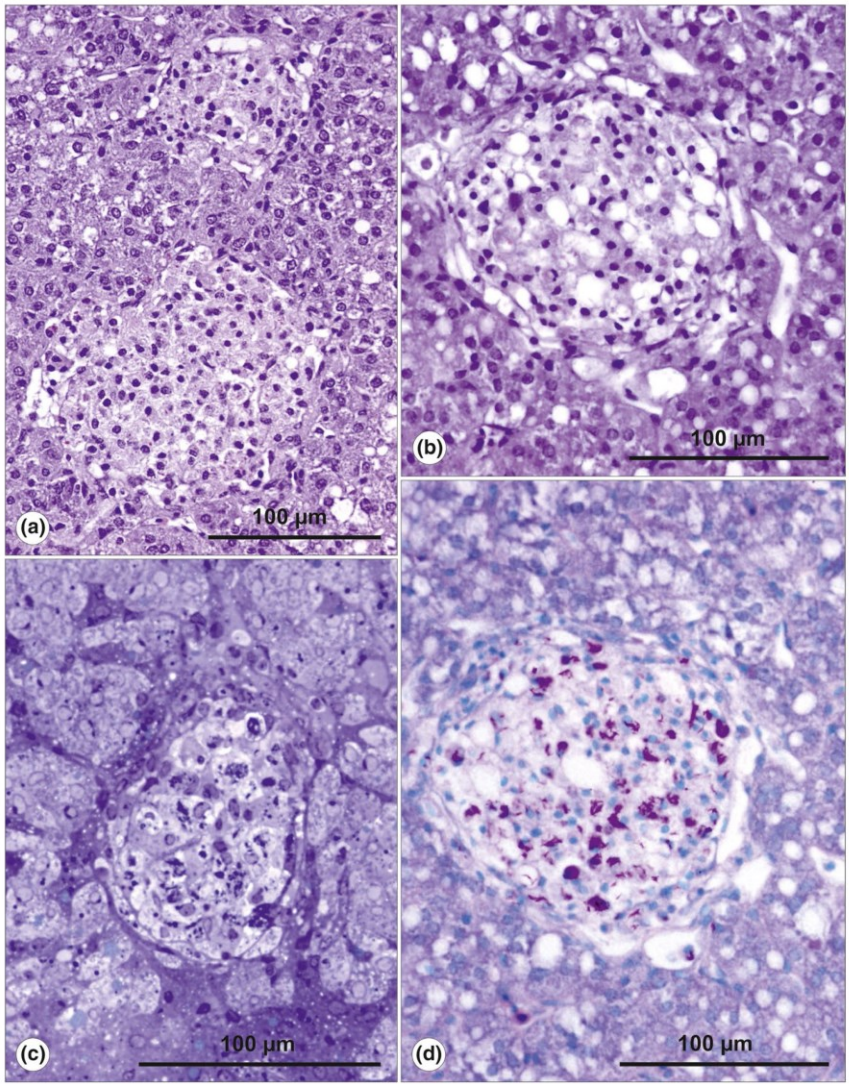


FIGURE 3 Liver parenchyma in GRZ strain of *Nothobranchius furzeri* infected with mycobacteria (a–d), invisible in H&E-stained monocyte/macrophage centres/cell aggregates (a,b), suspected in semithin section stained with toluidine blue (c), and well demonstrated in (d) section parallel to (b) that was stained with ZN

3.3 | Agents of mycobacterial infections

In kidney tissue samples of the 11*N. furzeri* (4 GRZ and 7 MZM), three species were determined. *Mycobacterium marinum* was determined in all 7 MZM and one (25%) GRZ samples based on identity of full-length 16S sequences with *M. marinum* CCUG 20998 and DSM 44344. Determination in the GRZ individual was confirmed by analysis of variable V3/V4 regions. In 3 (75%) GRZ individuals, more than 99.9% of 16S reads completely mapped to 16S rRNA of *M. chelonae* subsp. *gwanakae* (MOTT36W, the type strain) and of *M. saopaulense* (ID EPM 10906 and CCUG 66554).

4 | DISCUSSION

Histopathological lesions observed in our assemblage of *Nothobranchius* fishes were relatively easy to classify as either non-proliferative or proliferative. The interpretation of lesions within each of these categories was more difficult and required a careful evaluation of findings, comparison with lesions described in previous papers on histopathology of *Nothobranchius* and other fish species, and also selection of appropriate diagnostic terminology consistent with internationally harmonized nomenclature and diagnostic criteria for rat and mouse (Keenan et al., 2015; Thoolen et al., 2010) and fish (Boorman et al., 1997; Wolf et al., 2015; Wolf & Wolfe, 2005).

The interpretation of the predominating non-proliferative lesion, that is the accumulation of lipids and glycogen in hepatocytes, remained open for several reasons. In general, fish accumulate considerably more lipids/glycogen than mammals (Wolf & Wolfe, 2005) and the energy demands of annual killifish have not been specified for different periods of their short life, although sex-related differences were documented (Di Cicco et al., 2011; Vrtílek et al., 2018). The usual level of lipid accumulation observed in hepatocytes of *Nothobranchius* fishes would be classified into other fish species as dystrophy/degenerative pathological process. Considering that a high level of lipid storage was also described in individuals from wild populations (Vrtílek et al., 2018), the border between the natural/physiological condition and a pathological process is difficult to determine. Recently, the hepatocellular accumulation of foreign materials has received attention in connection with attempts to standardize the *Nothobranchius* fish diet (Žák et al., 2020).

Light microscopically discernible lesions in the kidney, spleen and liver of *Nothobranchius* fishes had in common the proliferation of mononuclear cells which either formed aggregates strikingly reminiscent of macrophage centres (MC) (predominantly in the liver) or proliferated diffusely (mostly in the kidney interstitium). Similar changes were described in earlier studies (Cooper et al., 1983; Markofsky, 1979; Markofsky & Milstoc, 1979) as "nodular degeneration progressing to cyst-like formations" (in the liver) and "nodular type of histiocytic lymphoma" (in the kidney). Aggregates of mononuclear macrophages (MAs), light microscopically identical with those documented by authors in the last decade (Blažek et al., 2017; Cellerino et al., 2016; Di Cicco et al., 2011), were presented earlier in an overview on medaka lesions induced in a carcinogenetic study (Boorman et al., 1997). In the latter study, the presence of MAs in the liver was termed "focal histiocytosis"—however, a clear distinction between the MAs and the hepatic granulomas was not presented (see figure 7 in Boorman et al., 1997, where an aggregate of macrophages is called a hepatic granuloma). A convincing example that the MAs can be distinguished from the granulomas in the fish liver was given by Wolf and Wolf (2005).

A comparison of the data obtained from the material newly collected by ourselves, those obtained by our re-examination of the material archived from the study by Blažek et al. (2017) and those published by Cooper et al. (1983), Markofsky (1979) and Markofsky and Milstoc (1979), showed that all these studies dealt with an identical type of cell aggregates in the liver, which were classified as hepatomas and hepatocarcinomas by Di Cicco et al. (2011) and later adopted in other studies (Blažek et al., 2017; Cellerino et al., 2016; Godoy et al., 2019; Součková et al., 2018). We suggest designating the cell aggregates of this type mononuclear cell aggregates (MAs) because there is no evidence of their hepatocellular origin (cf., Goodman, 2007; Hruban, 1979; Quaglia, 2018). In histopathological evaluation of proliferating cells, we decided to keep to the Wittamer et al. (2011) terminology, leaving a more detailed determination of monomorphic mononuclear cells of low-level differentiation to immunogenetic markers of a further study. We believe that this approach better corresponds to the results that could potentially be obtained on the cell types of *Nothobranchius* fishes using the light microscope.

Although the results of our study and comparison with published data prompt us to present a different opinion on the nature of the lesions, all the above-mentioned studies strongly influenced our lesion assessment approach, especially in the initial stage of research. We took into consideration non-neoplastic processes related to hematologic and immune competency of ageing fish and neoplastic processes triggered by an antigenic impulse, specifically case studies on the aetiology of plasmacytoid leukaemia in netpen-reared and wild-caught Chinook salmon (*Oncorhynchus tshawytscha*). These studies demonstrated the complexity of diagnostics and limits of histology in diagnosis of this leukaemia (Eaton et al., 1994; Eaton & Kent, 1992; Kent & Dawe, 1993; Stephen et al., 1995). The safe determination of virus aetiology of this disease required the exclusion of other suspected agents that were associated with histopathological lesions and, finally, the experimental verification of viral aetiology. Despite the dissimilarity of the fish species studied, the disease conditions histologically proven in Chinook salmon do not appear to be different from our *Nothobranchius* study. In both taxa, Chinook salmon and *Nothobranchius* fishes, the proliferation of mononuclear blasts occurred in both the haemopoietic organs and a non-haemopoietic one, suggesting leukaemia type of proliferation. The suspected neoplastic process observed in *Nothobranchius* H&E-stained sections resembled mononuclear cell leukaemia (MNCL) described in aged rats of the inbred F-344 strain by Caldwell (1999) and also mentioned in the review of splenic histopathology by Suttie (2006).

Histological examination plays a pivotal role in the accurate diagnosis of many disease processes including some tumours. In the diagnosis of others, for example, haematopoietic neoplastic diseases, histology is seriously limited. Its informative value has improved but not changed substantially since Harshbarger (1984) analysed similarities between certain non-neoplastic lesions/pseudoneoplasias and neoplasms in fish, amphibians and reptiles at The Symposium on the Use of Small Fish Species in Carcinogenicity Testing (Bethesda, MD, December 8–10, 1981). Immunohistochemistry can support histological diagnoses and is widely used in traditional animal models and human oncology but faces difficulties in fish due to the lack of appropriate markers, antibodies and reference sections.

The combined presence of the neoplasms, bacteria and viruses was widely discussed in a comprehensive review on neoplasms of the urinary tract in fish by Lombardini et al. (2014) and a study by Burns et al. (2018). These authors drew attention to infectious aetiologies of neoplastic proliferations and agents suspected of inducing tumours in fish. Association of chronic fish mycobacterial infections with cancer was tested by Broussard et al. (2009) in a series of experimental *M. marinum* infections in Japanese medaka. *M. marinum* infections only acted as promoters potentiating hepatocellular proliferative lesions when supported with low doses of the mutagen benzo[a]pyrene. To date, neither mycobacterial infections of *Nothobranchius* fishes nor their association with neoplasias (as described previously) have been studied systematically.

Our detection of rod-shaped bacteria in ultrathin sections of MZM individuals led us to recognize the limits of H&E staining in detection of suspected agents of lesions and focus on the ZN staining method that facilitated recognition of mycobacteria as agents of lesions. This staining dispelled our remaining uncertainties about the nature of mononuclear cells in the liver aggregates, allowed us to classify them as phagocytic macrophages and, despite the low level of pigmentation, assign aggregates of these cells to the category of melano-macrophage centres studied in detail in other bony fishes for almost 50 years (Agius, 1985; Roberts, 1978; Steinel & Bolnick, 2017; Wolke, 1992).

Great interest in fish mycobacterioses evidenced by the large number of original papers and specifically focused reviews permits the analysis of our results from several perspectives. Of these, the type of host response is the most important. The reaction of interstitial kidney tissue associated with mycobacterial infections in the GRZ assemblage does not differ from bacterial kidney disease (BKD) in Atlantic Salmon (Bruno et al., 2013; Noga, 2006). As in BKD caused by the diplobacillus *Renibacterium salmoninarum*, the proliferation of haematopoietic kidney tissue caused by mycobacteria is considerable and has a substantial indirect effect on the excretory structures of this organ. Injury caused by the host's reaction, mentioned previously by Thompson (1984), has also been observed in an advanced stage of mixed *Glugea–Mycobacterium* infections of *Nothobranchius* fishes obtained from hobby fish breeder (unpublished).

The comparison of GRZ infections with data accumulated on spontaneous mycobacterial infections in other bony fishes shows considerable similarity with those of captive syngnathids (Fogelson et al., 2017). Host reactions in syngnathid fishes were dominated by macrophages and "atypical" inflammatory reactions sensu Gauthier et al. (2003) or "defective" inflammatory reactions sensu Fogelson et al. (2017). Special terms were used to emphasize that inflammatory reactions differ from typical chronic granulomas described in other teleosts including zebrafish (Kent et al., 2004) in the absence of concentrically arranged connective tissue/spindle-cell capsule demarcating the necrotic core of the lesion from surrounding tissue. By understanding histopathological changes as indicators of immune functions in vertebrates (including fish), we can assume that manifestations of innate immunity dominated in short-lived *Nothobranchius* fishes, whereas adaptive immune cells probably did not receive strong enough signals to induce a typical granulomatous inflammatory reaction.

Spontaneous and experimental mycobacterial infections in fish are not easy to compare, because the entry routes of infection are different (spontaneous/natural vs. intramuscular or intraperitoneal) and also infectious doses differ (unknown vs. precisely calculated doses of agents administered in experiments). Comparison of our findings with published studies demonstrates an exceptionally high level of mycobacterial infectivity, that is transmissibility and the ability of mycobacteria to reproduce in the organs of *Nothobranchius* fishes. Whether or not these characteristics are specific to *Mycobacterium* species that contaminate the *Nothobranchius* breeding system remains to be determined. Compared with the ample data published on mycobacteria in zebrafish colonies (Kent et al., 2004; Watral & Kent, 2007; Whipps et al., 2012), our *Mycobacterium* species determination in 11 fish provides only a preliminary indication of possible mycobacterial agents. We intend to make an extensive determination of *Mycobacterium* species in our large set of cryopreserved tissue samples of both *Nothobranchius* strains (MZM and GRZ) of this study, expected to bring representative data for comparison with the above-mentioned comprehensive studies in zebrafish colonies.

The potential role of mycobacteria as promoters of neoplastic proliferation, as well as the possibly enhanced susceptibility to infection of inbred fish lines (such as GRZ) requires further investigation.

Considering the ubiquity of non-tuberculous mycobacteria, our results demonstrate the importance of preventing their introduction into *Nothobranchius* breeding facilities by any means. This includes preventing infection from biofilms on aquarium sides by careful disinfection of aquaria prior to fish introduction (Chang et al., 2018), disinfection of eggs prior to hatching (Chang et al., 2015), elimination of alimentary contamination by live or defrosted food (Chang et al., 2019; Nenoff & Uhlemann, 2006) and the development and use of practical dry food for *Nothobranchius* fish (Žák et al., 2020). Examples of numerous additional measures are detailed in Mason et al. (2016).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets used for the current study are available from Iva Dyková (histopathology), Jakub Žák (statistical analysis) and Kamila Součková (molecular analysis) upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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DISCUSSION

This dissertation examined a variety of topics relating to the biology and senescence of *Nothobranchius* fish. Life history traits from captive and wild populations were interconnected and were used for designing experiments focused on the environmental control of senescence and age related deterioration of reproductive capacity. This dissertation emphasized that knowledge of the natural biology of model organisms is extremely important for correct experimental design as well as for proper interpretation of laboratory outcomes. Further, the life history of *Nothobranchius* was placed in the broader context of short-lived ray-finned fishes, the most diverse lineage of vertebrates with such short lifespans. A much deeper discussion about each topic was presented in the attached articles above, therefore, each topic is only briefly discussed below and interrelated with other works which are part of this dissertation.

An important baseline for the multiple papers presented in this dissertation is research on senescence-related life history traits of wild *Nothobranchius*. The extreme pace to maturation (Paper 2) and exceptionally fast growth of wild fish contrasts with observations repeatedly made in captivity (e.g. Paper 3). In addition to this, the thesis also presents the first data on locomotor activity in the natural environment (Paper 7). All the mentioned traits have a significant impact on senescence (maturity: Markofsky & Perlmutter, 1972; growth: Lee et al., 2013) or are used as markers of senescence (locomotor activity: Lucas-Sánchez et al., 2015; Almaida-Pagán et al., 2018). Knowledge from the wild is therefore essential for proper interpretation of findings from laboratory experiments.

Investigation of reproductive senescence in the wild (Paper 5) and captivity (Paper 6) provided contrasting results. Apparent absence of reproductive senescence in the wild population and its expression later in life when *N. furzeri* is kept in protected conditions is not that surprising. The fish do not have enough time to senesce in the ephemeral pool, but when

protected from premature death their reproductive functions clearly deteriorate at advanced age. The results from our research on wild and captive *Nothobranchius furzeri* support the theory of Medawar, which predicts that senescence may express only when extrinsic mortality is suddenly reduced (Medawar, 1952), explaining why senescent individuals are extremely rare in the wild. Recent molecular studies support an accumulation of deleterious mutations as the central mechanism governing senescence in *N. furzeri* (Cui et al., 2019; Willemsen et al., 2020). We now possess a relatively complete picture of the overall pattern of reproductive senescence of *N. furzeri*. However, specific molecular mechanisms (e.g. gene expression) behind the age-related decline in reproduction of *N. furzeri* remain to be investigated.

Using the experimental data on preferred temperature of *Nothobranchius* fish (Paper 8), together with observations on diurnal fluctuations in their natural environment and behaviour (Paper 7), allowed us to set up ecologically relevant temperature fluctuations in laboratory during a whole lifespan of *N. furzeri* (Paper 9). The ecologically relevant thermal fluctuation resulted in better survival and increased relative fecundity in the lab. The study also showed that previous experimental results, under constant temperature, may be biased, this also applies to some studies that are part of this thesis. An experiment examining the effect of thermal fluctuation on senescence has probably never been conducted in other vertebrates and points to environmental intervention as a potential trigger for senescence in vertebrates.

Laboratory breeding inevitably entails some artificial conditions not occurring in the natural environment. For example, the captive environment may contribute to an unnaturally high incidence of neoplasias from inappropriate nutrition (Spitsbergen et al., 2012) or the effect of pathogens (Hochberg & Noble, 2017), this also applies to *N. furzeri* in laboratories. Our data from 137 livers of *N. furzeri* at ages between 17 – 121 days collected from the wild showed neoplasias in only two individuals (unpublished data). Paper 11 proposes that the

most important factor behind high "tumour" incidence in some studies on *Nothobranchius* spp. published in the last two decades was chronic bacterial infection followed by misdiagnoses of the altered tissue (Wolf et al., 2015, Paper 11). Whether the high incidence of immune responses in advanced age is a symptom of inflamm-aging (Franceschi et al., 2000) or if it is related to time dependent bacterial/viral load in the body/environment causing chronic disease, is an open question. In future research, more attention should be devoted to proper identification of histological and histopathological findings.

The findings presented here open new avenues for experimental research using short lived killifish as a model. For example, the protocol enabling easy transition of *Nothobranchius* fish from live food to a pelleted diet provides an opportunity for dietary intervention of senescence via macronutrient manipulation. Additionally, this allows killifish research to be more rigorous by potentially reducing among-laboratory variability in husbandry conditions. The ability of killifish to accept dry feed will hopefully encourage researchers to experiment with the essential nutritional requirements of this species and ultimately, to provide a standardized diet that could be used across laboratories completely avoiding risky live and frozen food in *Nothobranchius* husbandry.

This dissertation confirms the important position of newly emerging model organisms in senescence research thanks to their practicality. In contrast to fruit flies or mice, *Nothobranchius* fish are useful for conducting research on senescence in wild populations. There is limited risk of emigration of *Nothobranchius* spp. from a studied population due to their presence in area-restricted small pools enabling satisfactory recapture rates. In addition, *Nothobranchius* fish hatch synchronously (Polačik et al., 2011) and have non-overlapping generations. These traits, together with their exceptionally short lifespan have been fully exploited by this dissertation project. The majority of studies comparing senescence between the natural environment and captive conditions are not usually performed on the traditionally

used animal models (e.g. fly *Telostylinus angusticollis*: Kawasaki et al., 2008; large herbivores: Lemaître et al., 2013; lemur *Microcebus murinus*: Hämäläinen et al., 2014; monkey *Aotus azarae*: Larson et al., 2016; antler fly *Protopiophila litigata*: Mautz et al., 2019). In this respect, despite bring a recently established model organism, *N. furzeri* seems to be exceptional because its senescence has been studied both in the captivity and in the wild (Smith et al., 2017, Papers 5 & 6). This dissertation project emphasizes that a combination of research in the natural (original) habitat and in controlled conditions in a laboratory is critical and fruitful. It is the only way to ascertain that laboriously collected data are not just artefacts of the captive conditions.

The major impact of this dissertation is threefold. 1/ it indisputably presents empirical data on reproductive senescence in fish – a vertebrate with a long period of post-maturity growth; 2/ it significantly contributes to knowledge about environmental control of senescence – the role of ecologically relevant fluctuating temperature and the influence of natural *vs* captive conditions on reproductive senescence; 3/ it introduces the more rigorous use of *Nothobranchius* fish in laboratory research by presenting a protocol for replacing live food with pellets, as well as identifying misdiagnoses in histopathology of this species. Some researchers may consider other outcomes more important, for example, a description of the earliest age at maturation found in any vertebrate or evidencing the diversity of fish species with natural lifespan shorter than one year. I chose the above mentioned points as the most important because they challenged previously postulated hypotheses or common practice in research using *Nothobranchius* fishes as models and may have direct consequences for *Nothobranchius* research. In conclusion, this dissertation emphasizes that the environment significantly modulates senescence and that a combination of laboratory and field research as well as essential knowledge of the biology of model organisms is extremely important for designing experiments and interpreting their outcomes.

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ANNEX

These works were published during the course of the PhD study and are not part of the dissertation.

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