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RESEARCH ARTICLE

The sources of sex differences in aging in annual fishes

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Abstract

1. Intersexual differences in life span (age at death) and aging (increase in mortality risk associated with functional deterioration) are widespread among animals, from nematodes to humans. Males often live shorter than females, but there is substantial unexplained variation among species and populations. Despite extensive research, it is poorly understood how life span differences between the sexes are modulated by an interplay among genetic, environmental and social factors.
2. The goal of our study was to test how sex differences in life span and ageing are modulated by social and environmental factors, and by intrinsic differences between males and females.
3. To disentangle the complex basis of sex differences in life span and aging, we combined comparative data from sex ratios in 367 natural populations of four species of African annual killifish with experimental results on sex differences in life span and aging from eight laboratory populations tested in treatments that varied social and environmental conditions.
4. In the wild, females consistently outlived males. In captivity, sex-specific mortality depended on social conditions. In social-housed experimental groups, male-biased mortality persisted in two aggressive species, but ceased in two placid species. When social and physical contacts were prevented by housing all fish individually, male-biased mortality ceased in all four species. This outcome held across benign and challenging environmental conditions. Fitting demographic survival models revealed that increased baseline mortality was primarily responsible for a shorter male life span in social-housing conditions. The timing and rate of aging were not different between the sexes. No marker of functional aging we recorded in our study (lipofuscin accumulation, proliferative changes in kidney and liver) differed between males and females, despite their previously confirmed association with functional aging in *Nothobranchius* killifish.

5. We show that sex differences in life span and aging in killifish are driven by a combination of social and environmental conditions, rather than differential functional aging. They are primarily linked to sexual selection but precipitated through multiple processes (predation, social interference). This demonstrates how sex-specific mortality varies among species even within an ecologically and evolutionary discrete lineage and explains how external factors mediate this difference.

KEYWORDS
adult sex ratio, demographic ageing, gender, *Nothobranchius furzeri*, predation, senescence, social environment

1 | INTRODUCTION

Males and females differ in many demographic and life-history parameters, with important consequences for ecology, evolution and physiology, as well as for practical and societal outcomes (Austad, 2006; Regan & Partridge, 2013). Intersexual differences in life span (age at death) and aging (increase in mortality risk associated with deterioration in bodily functions) are widespread among animals, from nematodes to humans (Austad & Fischer, 2016). Males are usually the shorter lived sex (Lemaître et al., 2020; Liker & Székely, 2005; Promislow, 2003), but there is substantial unexplained variation among species and populations (Austad & Fischer, 2016). Intersexual differences in life span and aging appear modulated by environmental and social factors (Austad, 2006; Lemaître et al., 2020), but their effects remain opaque.

Why do males typically express a truncated life span in comparison with females? One set of explanations posits that the primary difference stems from genetic and genomic differences between the sexes (Gemmell et al., 2004; Maklakov & Lummaa, 2013). In mammals, fruit flies and many other taxa, males are the heterogametic sex and the hemizygosity of key genes located on the sex chromosomes resulting in an inability to compensate for the effects of deleterious mutations has been implicated in shorter male life span (Trivers, 1972; Xirocostas et al., 2020). However, males also show shorter life spans in many birds and butterflies, despite female heterogamy in those groups (Gotthard et al., 2000; Sielezniew et al., 2020; Tompkins & Anderson, 2019). Asymmetry in the inheritance of mitochondria, leading to suboptimal compatibility between mitochondrial and nuclear genomes in males (Frank & Hurst, 1996), could explain male-biased mortality and aging in heterogametic taxa (Gemmell et al., 2004). Yet, these explanations cannot account for the wide variation in the sex bias in life span and aging rate seen among populations of a species and among inbred laboratory strains housed under contrasting conditions (Austad & Fischer, 2016), strongly implicating other factors in driving sex-biased mortality.

Males and females differ in their routes to reproductive success. These divergent trajectories arise as a consequence of gamete size disparity which leads to variation in the reproductive roles of males and females (Fromhage & Jennions, 2016). This disparity is best explained by sexual selection, with asymmetric variation in reproductive success between the sexes (Andersson, 1994). Male reproductive success is often skewed towards a few highly successful individuals, while female reproductive success is far less variable (Clutton-Brock, 2021). Mating system is a key modulator of this variation. In a monogamous mating system, differences in the variance in reproductive success between males and females are often trivial. In highly polygynous mating systems, such as those with male harems, a single male may monopolize a large number of females generating highly skewed male reproductive success (Clutton-Brock, 2021).

Sexual selection is associated with elevated mortality in the more competitive sex (Székely et al., 2014). Conspicuous signalling to rivals and potential partners directly increases the risk of mortality from predators (Tuttle & Ryan, 1981). Male–male competition is also risky and may lead to increased mortality (Beirne et al., 2015). Higher male mortality is often precipitated via alterations to hormonal profiles, resulting in chronic stress (Keller et al., 1992) or elevated testosterone levels (Foo et al., 2017), thereby making individuals more susceptible to infections or physiological deterioration (Gupta et al., 2020; Moore & Wilson, 2002).

African annual fishes from the genus *Nothobranchius* are a taxon ideally suited to biomedical and evolutionary studies on aging (Cellerino et al., 2016; Cui et al., 2019; Hu & Brunet, 2018). Inhabiting ephemeral savanna pools, they have evolved naturally short life spans which recapitulate typical features of vertebrate aging, including multifarious functional deterioration in old age (Cellerino et al., 2016; Hu & Brunet, 2018). In the wild, killifish hatch at the onset of the rainy season from desiccation-resistant eggs. Both sexes grow rapidly and achieve sexual maturity in as few as 2 weeks (Vrtílek et al., 2018). Males compete for access to females, with a marked variability in the strength of intra-sexual competition among species (Cellerino et al., 2016; Genade, 2005; Polačik & Reichard, 2011; Wildekamp, 2004). Male–male aggressive interactions include frontal and lateral displays and often escalate into physical aggression between two males, including biting the opponent (Reichard, 2015). Although their natural life span is limited by desiccation of their habitat, most fish (of both sexes) succumb long before their natal pool desiccates (Vrtílek et al., 2018). Their short life span of several months is retained in captivity, where fish are

shielded from extrinsic mortality, with captive fish suffering a range of functional declines, including increasing incidence and severity of proliferative changes leading to organ dysfunction and accumulation of lipofuscin in post-mitotic cells (Cellerino et al., 2016). In all *Nothobranchius* species for which information on sex chromosomes is available, males are the heterogametic sex, and sex chromosomes are morphologically distinguishable in at least eight species (Krysanov & Demidova, 2018; Reichwald et al., 2015). In this study, we used four species of annual killifish from Southern and Central Mozambique: *Nothobranchius furzeri*, *N. orthonotus*, *N. pienaari* and *N. kadleci* (Reichard et al., 2017).

To disentangle the complex basis of sex differences in life span and aging, we combined sex ratio estimates from 376 wild populations with experimental data from replicated laboratory populations tested in treatments that varied social and environmental conditions. First, we compared sex ratios in captive populations estimated at the onset of sexual maturity with the sex ratios in wild populations collected throughout adulthood to examine the role of natural conditions (including predation) on sex-biased mortality. Second, we used eight captive populations (e.g. excluding predation) investigated the role of social environment in sex-biased mortality and compared life span and aging between fish kept in social groups and those housed individually. When the sexes differed in life span, we compared demographic aging parameters between males and females to understand the sources of this difference (background mortality or aging rate). Third, we subjected fish to fluctuating temperature to test whether sex-biased mortality is related to challenging environmental conditions that fish experience in the wild, but not in laboratory. Finally, we compared three biomarkers of functional aging between males and females at young and old ages.

We expected female-biased adult sex ratios in the wild due to sex-biased predation (Reichard et al., 2014). The disappearance of sex-biased mortality in captivity (irrespective of social and environmental treatment) would indicate the major role of predation. Its disappearance in individual (compared to social) housing would suggest the role of male–male competition in male-biased mortality. Finally, the presence of sex-specific aging at functional level (and the reappearance of sex-biased mortality in the challenging environment) would indicate intrinsically greater male fragility.

2 | MATERIALS AND METHODS

2.1 | Sex ratio estimates

To quantify sex ratios at the start of sexual maturity, we used data on sex ratios from 63 cohorts of outbred, wild-derived captive populations of all four study species in protected laboratory conditions, collected at the onset of adulthood (age 3–5 weeks) over a period of several years. Within each cohort, fish were hatched on the same day, following standard husbandry protocol (Polačik et al., 2016). Their sex ratios were estimated once all fish attained sexual maturity (4–5 weeks after hatching) and their sex was phenotypically unambiguous.

The sex ratio in wild populations of all four study species was estimated during 10 field trips to Mozambique conducted between 2008 and 2015. *Nothobranchius* populations contain a single age cohort since fish hatch in synchrony soon after the rains fill their natal pools. Their population densities were estimated to range from 0.03 and 3.3 individuals per m^2 (Vrtílek, Žák, Polačik, et al., 2018). The age of fish when sex ratios were estimated was unknown, but varied from the onset of adulthood to later age. At each site, fish were collected using a triangular dip net (45 × 45 cm, mesh size 5 mm) or beach seine (length 2.7 m, depth 0.7 m, mesh size 4 mm). The method retained adult killifish unselectively and there was no sex bias in the probability of capture, confirmed by a combination of capture–mark–recapture studies and removal sampling (Reichard et al., 2014; Vrtílek, Žák, Polačik, et al., 2018). Fish were sorted into species and sexed on the bank, counted and released back to the pool. Details of data collection are provided in Reichard et al. (2014); the new samples used in the present study were collected following an identical protocol. We only used estimates based on at least six individuals of a given species in further analyses. This yielded adult sex ratios from 376 wild populations (based on a total of 15,968 fish).

Sex ratios were analysed using a generalized linear mixed model (GLMM) with binomial error structure (male to female ratio) and logit-link function in the LME4 package (Bates et al., 2015), where *Species* was treated as a fixed factor and *Year* and *Site* as random factors.

2.2 | Experimental populations

For laboratory experiments, we used fish from four related *Nothobranchius* species from southern and central Mozambique (Reichard et al., 2017). Each species was represented by two independent populations, originating from separate intraspecific lineages (Bartáková et al., 2015). Experimental fish were F1 descendants of wild parents collected in Mozambique. The locations of source populations are presented in Table S1. Eggs of parental fish were stored in an incubator (Pollab, Q-CELL 60-240) at 24 ± 0.5°C for at least 16 weeks following standard husbandry protocols (Polačik et al., 2016). The experiment was divided into two phases for logistical reasons (capacity of experimental facility). Work on *N. furzeri* and *N. kadleci* was conducted from September 2011 to December 2012, followed by work on *N. orthonotus* and *N. pienaari* (May 2013–March 2015).

Experimental fish were hatched simultaneously by watering the incubation substrate with dechlorinated tap water (16°C). From the age of 2–10 days (depending on size of the juveniles, but before the sexes could be separated), fish were housed either in social tanks (24 L) or individually (2 L tanks), providing identical fish density (number of fish per volume of water) between treatments. During the juvenile period, any dead experimental fish were replaced with fish of the same age and housing history from

stock tanks. At the age of 6–7 weeks, fish in social tanks were marked with a single Visible Implant Elastomer tag (Northwestern Marine Technology) to enable individual recognition, except for *N. pienaari* due to its smaller body size. Previous studies have shown no negative effect of marking on subsequent survival (Sandford et al., 2020). Nine to twelve tanks were used for each study population (total of 84 groups), with initial density of 12 fish per 24 L tank, except for *N. orthonotus* (the largest species) where the density was 10 fish per tank. In social tanks, male–male aggressive interactions were common. Water quality was maintained using air-driven filters and 25%–30% of water was exchanged every 2–3 days. Individually housed fish were kept in 2 L tanks in two separate recirculating systems (Aquamedic, Germany, www.aquamedic.de), with 45 fish per species (22–23 fish per population). All fish were kept under a 12 hr:12 hr light:dark regime in aged tap water (conductivity 550 μS/cm), at a water temperature of 26 ± 2°C. Fish were fed twice each day to satiation during the first month and once a day thereafter. Fish were initially fed with live *Artemia* nauplii and weaned to chopped bloodworm (*Chironomus* larvae) and *Tubifex* from the age of 10–30 days. All tanks received the same ration (approximately 15% of body mass of the fish in the tank).

2.3 | Environmental challenge

One possible explanation of failure to detect higher male mortality in captivity (despite its occurrence in the wild) can arise from unnaturally benign laboratory conditions, compared to a challenging natural environment. To test the effect of unfavourable environmental conditions arising from fluctuating temperature (Thomas et al., 1986) on sex-specific survival, we compared sex differences in life span in a cohort of individually housed fish (*N. furzeri*, population A) that experienced either a stable temperature ($M \pm SD$: 27.5 ± 1°C; control fish) or fluctuating temperature (from 20 ± 1°C in early morning to 35 ± 1°C in late afternoon). The limits for the fluctuating temperature reflected the diurnal change in water temperature that killifish typically experience in the wild (Žák et al., 2018). This thermal challenge is not employed in the standard breeding protocol for captive killifish (Polačik et al., 2016). A fluctuating temperature was achieved 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). The stable temperature in the control group was regulated by one 100 W heater.

2.4 | Life span estimates

All tanks were monitored daily for dead fish. Survival was estimated from the age when all fish of a given species were sexually mature (5 weeks in *N. orthonotus*, 6 weeks in *N. furzeri* and *N. kadleci*, 8 weeks in *N. pienaari*). Sex differences in mortality were analysed using species-specific mixed effects Cox proportional-hazards models (coxme package, likelihood ratio tests) (Therneau, 2015) with Sex as a fixed factor and Population as a random factor. Note that analysis using Population as the fixed factor (*coxph* function in survival package, based on likelihood ratio tests, Wald test or log-rank tests, and including population by sex interaction) generated an identical interpretation. Analyses were completed separately for each species and social environment. Fish removed from social tanks for the analysis of functional aging were censored in the survival analysis at the age of removal. Sample size for all analyses is given in Table S1.

2.5 | Actuarial aging

Demographically, sex-biased life span can arise from differences in baseline mortality (i.e. one sex experiencing persistently higher mortality throughout adulthood) or rate of aging (i.e. a steeper increase in mortality with age in one sex). These two sources of differential mortality can be estimated with a Gompertz model of increasing failure time (Boonekamp et al., 2020; Bronikowski et al., 2011), which provide clear demographic interpretation of the parameters.

To test whether Gompertz-family models well approximated the observed mortality patterns and to estimate intersexual differences in actuarial ageing within species with sex-biased mortality, we used Bayesian survival trajectory analysis (BaSTA package; Colchero et al., 2012). First, we fitted a set of demographic models using a function *multibasta* (Weibull, Gompertz, Gompertz logistic), each with three possible shape parameterizations (simple, Makeham, Siler/bathtub) to our data from the social treatment, in which shorter male life span was detected. We then compared their fits using deviance information criterion (DIC), a Bayesian equivalent of Akaike information criterion (Table S2). We used four runs of each model, each with 150,000 MCMC iterations, burn-in of 15,000 and thinning by sampling every 50th estimate. For other parameters, we used default BaSTA setting (i.e. argument *covarsStruct* was set to 'fused'). We analysed each population separately as we knew a priori that populations within species differ in life span (Blažek et al., 2017) and, in contrast to survival analysis, they cannot be entered as random effects using BaSTA. Within species, the sex differences were compared using the same function and parametrization across both populations.

Gompertz-family models provided good fit to our datasets (Table S2) and allow comparable demographic interpretations across study populations. The Gompertz model assumes that aging starts at a species-specific age, with one parameter (intercept, *Initial mortality rate*, *IMR*) describing age-independent mortality (baseline mortality) and the second parameter (slope, *Rate of Aging*, *RoA*) describing the increase in mortality with age (Pletcher et al., 2000). In both *N. furzeri* populations, deceleration in aging was apparent at old age, probably arising from intra-population heterogeneity (Chen et al., 2013), and Gompertz-logistic models were used as their DIC was considerably lower than a simple Gompertz model (Table S2). A Gompertz-logistic model estimates a third parameter (*s*) which

models deceleration of aging rate at old age. The final models were run with 400,000 MCMC iterations, burn-in of 50,000 and thinning by sampling every 50th estimate to provide a posterior distribution of parameters for each species. Model parameters were compared between males and females using the Kullback–Leibler discrepancy criterion (KL). The KL varies between 0.5 (complete overlap) and 1.0 (no overlap) with values >0.8 being considered as a substantial difference (Colchero et al., 2012).

2.6 | Histopathology

In a protected environment, mortality derives from deterioration in bodily function. We contrasted data on biomarkers of cellular and physiological aging between males and females from four experimental populations of *N. furzeri* and *N. kadleci*, kept in the social treatment. Subsamples of young (age 14 weeks) and old (age 23 weeks) *N. furzeri* and *N. kadleci* from social treatment were sacrificed and dissected (20 fish per age and species). The liver and kidneys were preserved in Baker's solution, embedded in Paraplast, sectioned (5 μm) and stained in H&E. From the histological slides, the incidence of proliferative changes was scored using a 5-grade pathological scale (Di Cicco et al., 2011) (score 0–4, 0: no proliferation, 4: >50% of tissue filled with proliferative cells). Data were analysed using cumulative link mixed models for ordinal data in the package *ordinal* (Christensen, 2019), with Sex, Age and their interaction as fixed factors, and Population ID as a random factor.

As a biomarker of cellular aging, we compared the deposition of lipofuscin in liver tissue in young and old male and female fish. Lipofuscin is an aggregate of oxidized proteins that accumulates in aged post-mitotic cells (Jung et al., 2007). The amount of lipofuscin particles was estimated from separate slides (unstained sections) using a Leica confocal fluorescent microscope. Nine slides were analysed for each individual. Excitation wavelength was set to 488 nm (confocal parameters such as pinhole, photo-multiplier and laser intensity were fixed). Images were imported to *imageJ* and the number of lipofuscin (fluorescing) particles counted. Data were analysed using Generalized Linear Mixed Models with Poisson error distribution and log-link function, with Sex, Age and their interaction as fixed factors, and Population ID and Individual ID as random effects. The sample sizes for all analyses are given in Table S1.

2.7 | Ethical and fieldwork approvals

Research adhered to all national and institutional guidelines and regulations. Fieldwork complied with legal regulations of Mozambique (collection licenses: DPPM/053/7.10/08, 175/154/IIP/2009/DARPE, DPPM/083/7.10/10, DPPM/330/7.10/10, DPPM/069/7.10/11, DPPM/088/7.10/12). Experimental work was approved by the Ethical Committee of the Institute of Vertebrate Biology (No. 163-12) and by Ministry of Agriculture (CZ 62760203) in accordance with legal regulations of the Czech Republic.

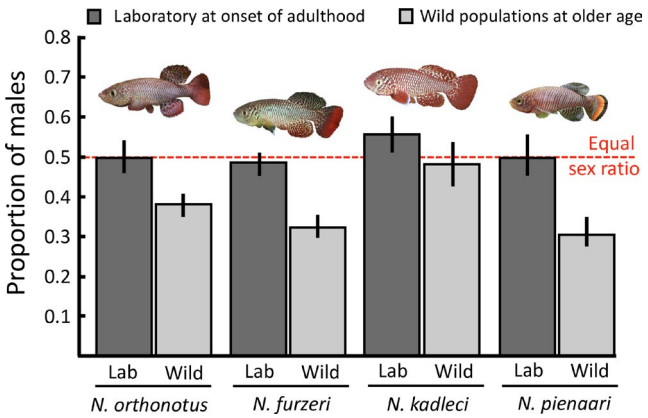

FIGURE 1 Proportion of males in wild-derived laboratory populations (grey columns, estimated at the onset of sexual maturity) and wild populations (green columns, estimated throughout adulthood with the age not known). Mean estimates and associated 95% confidence intervals, back-calculated from outcomes of binomial models, are shown. Species are ranked from the most aggressive (left) to the least aggressive (right)

3 | RESULTS

3.1 | Sex ratios in captive and wild populations

When fish were raised in protected captive conditions (n = 63 cohorts), sex ratios at the age of sexual maturation were equal in the three study species (GLMM with binomial error structure). The mean proportion of males (95% confidence intervals) was 48.9% (46.4%–51.5%) in *N. furzeri*, 49.6% (44.1%–55.0%) in *N. orthonotus* and 50.3% (44.7%–55.8%) in *N. pienaari*. The sex ratio in *N. kadleci* was significantly male-biased (55.8%, 52.2%–60.1%; Figure 1).

The estimates of sex ratios in natural populations were recorded at different phases of the adult life span, with the age of fish during sex ratio estimate being unknown, but typically weeks or months after reaching sexual maturity. In the three study species with equal sex ratio estimates in captivity, there were significantly more females than males in wild populations. The mean proportion of males (with 95% confidence intervals, calculated from GLMMs with binomial error structure) was 32.7% (30.0%–35.4%) in *N. furzeri*, 38.2% (35.3%–41.2%), in *N. orthonotus* and 30.9% (27.0%–35.0%) in *N. pienaari*. The sex ratio of *N. kadleci* (the species with male-biased sex ratio in captivity) was equal in wild populations (48.0%, 42.7%–53.5%) (Figure 1). This implies that mortality of adult males in natural populations was consistently higher than female mortality in all four species.

3.2 | Sex differences in life span—Social and environmental effects

To investigate how sex-biased mortality arises, we raised a set of killifish cohorts from a total of eight wild-derived populations from all four species (Table S1) in the laboratory and compared sex differences in life span and aging in two contrasting social treatments

TABLE 1 Sex-specific median life span estimates (with 95% confidence intervals estimated from the *survfit* function, in days) in the social and single housing treatments. NAs represent cases where the upper confidence interval cannot be reliably calculated due to limited sample size

		Median life span (95% CI)	
Species	**Sex**	**Social**	**Single**
Nothobranchius orthonotus	Females	256 (214–289)	232 (193–352)
	Males	180 (134–197)	271 (215–368)
Nothobranchius furzeri	Females	150 (131–176)	121 (104–NA)
	Males	121 (115–134)	117 (111–145)
Nothobranchius kadleci	Females	172 (148–272)	166 (103–240)
	Males	178 (161–206)	113 (97–NA)
Nothobranchius pienaari	Females	282 (221–338)	314 (242–347)
	Males	251 (227–286)	297 (235–446)

(group and individual fish housing). In social tanks (5–6 conspecific pairs housed together), male life spans were shorter in two aggressive species: *N. orthonotus* ($\chi^2 = 23.46$, $p < 0.001$; with male median life span being 42%, i.e. 76 days, shorter) and *N. furzeri* ($\chi^2 = 7.13$, $p = 0.008$; male life span 24%, i.e. 29 days, shorter). No difference in male and female life spans was found in two relatively more placid species, *N. kadleci* ($\chi^2 = 0.06$, $p = 0.810$) and *N. pienaari* ($\chi^2 = 0.07$, $p = 0.792$, Table 1).

When fish were housed singly, there was no sex difference in life span in any species (*N. orthonotus*: $\chi^2 = 0.02$, $p = 0.889$; *N. furzeri*: $\chi^2 = 0.25$, $p = 0.617$; *N. kadleci*: $\chi^2 = 1.59$, $p = 0.207$; *N. pienaari*: $\chi^2 = 2.20$, $p = 0.138$). Life span estimates of fish that lived in group-housed and singly housed treatments were congruent (95% confidence intervals for median life spans overlapped), except for an increase in median life span in singly housed male *N. orthonotus* (Table 1), the most aggressive species. This finding is consistent with the prediction that male–male aggression considerably decreased male life span in *N. orthonotus* in a group-housed social setting. In contrast, the lack of sex differences in life span in *N. furzeri* was also related to the decreased median life span in singly housed *N. furzeri* females. There was also (non-significant) numerical decrease in the life span of singly housed *N. kadleci* males.

Under challenging conditions (thermally fluctuating environment), there was no significant sex bias in mortality in singly housed *N. furzeri* ($\chi^2 = 3.46$, $p = 0.063$), with males (and not females) tending to live longer. Median life spans (and their 95% confidence intervals) were 364 (293–410) days in males and 290 (195–343) days in females. This trend was not observed under control stable temperature ($\chi^2 = 0.01$, $p = 0.926$; males: 230 (85–312) days, females: 152 (102–287) days). The longer life span in thermally fluctuating environment is addressed elsewhere (Žák & Reichard, 2020).

Overall, survival analysis supports the role of predation- and social stress-related truncation of male life span.

3.3 | Sex differences in actuarial aging

We used Bayesian survival trajectory analysis to estimate intersexual differences in actuarial ageing in species with sex-biased mortality in social housing (where sex differences were detected). In *N. orthonotus*, the species with the greatest contrast in life span between the sexes, male-biased mortality was the result of stronger baseline mortality in males rather than a higher rate of aging. This result was consistent across both study populations (Figure 2a,b). In *N. furzeri*, the second species with male-biased mortality in the social treatment, a Gompertz-logistic model (which includes a parameter for deceleration in the aging rate in old age) gave a considerably better fit to the observed data than a simple Gompertz model (Table S2). We detected lower mortality deceleration (parameter *c*) in one *N. furzeri* population (Figure 2c) and no intersexual difference in Gompertz-logistic model parameters in the second population (Figure 2d).

3.4 | Sex differences in functional aging

We contrasted data on biomarkers of functional aging between males and females from four experimental populations of *N. furzeri* and *N. kadleci*, kept in the social treatment and sampled at two age points. Lipofuscin deposition (and its increase with age) did not differ between males and females (GLMM with Poisson error distribution: sex: $z = 0.17$, $p = 0.862$; sex by age interaction: $z = 0.65$, $p = 0.514$; $n = 75$ fish; Table 2). Similarly, we found no intersexual differences in proliferative changes in the kidney (sex: $z = 1.71$, $p = 0.087$, sex by age interaction: $z = 1.16$, $p = 0.247$) and liver (sex: $z = 0.50$, $p = 0.615$, sex by age interaction: $z = 1.45$, $p = 0.147$) (Table 2). This suggests that the rate of functional aging, at least in the biomarkers used in our study, was not different between males and females.

4 | DISCUSSION

Intersexual differences in life span and aging are widespread among taxa, but despite a substantial research interest, it is still not clear how genetic differences between the sexes are modulated by environmental and social factors (Austad, 2006; Gordon et al., 2017; Lemaître et al., 2020). Using eight populations from four closely related annual killifish species, we combined comparative and experimental approaches to demonstrate that female bias in wild annual killifish populations arises from higher extrinsic male mortality linked to predation and social interactions, rather than from generalized intersexual differences in functional deterioration. Females consistently outlived males in the wild, but in captivity this difference persisted only in social tanks in more aggressive species, and ceased when fish were housed singly. Increased baseline mortality, rather than an earlier or faster rate of aging, was primarily responsible for the shorter male life span in a social setting. The same pattern is also found across human populations (Austad & Fischer, 2016).

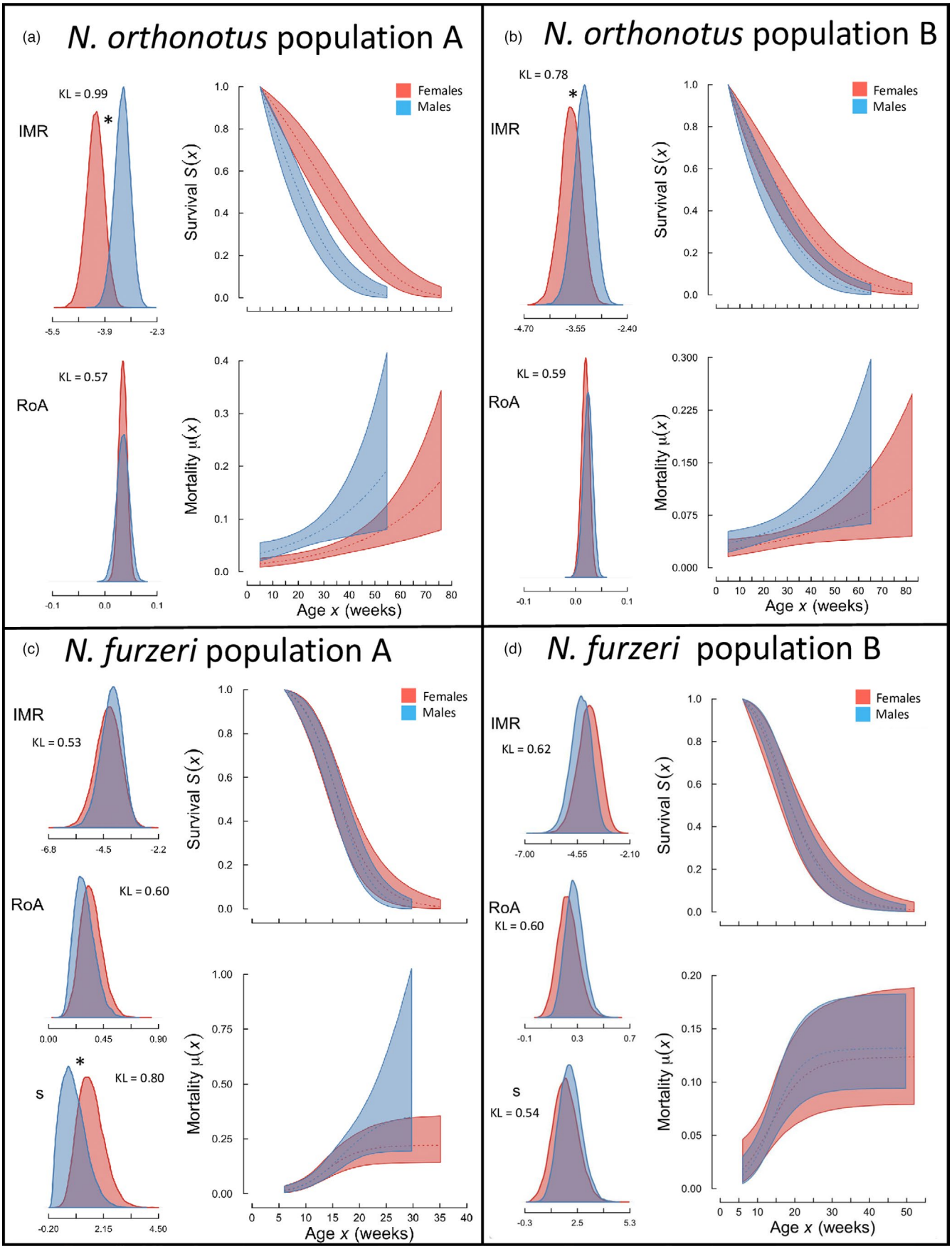

FIGURE 2 Posterior distribution of baseline mortality (IMR), rate of aging (RoA), survival and mortality risk estimated from Gompertz model for females (red) and males (blue) in two *Nothobranchius orthonotus* populations (a, b) and two *Nothobranchius furzeri* populations (c, d) in the social treatment. In *N. furzeri*, the posterior distribution for aging deceleration parameter (*s*) is also presented. KL denotes Kullback–Leibler discrepancy criterion, with values >0.8 considered as a substantial difference (marked by an asterisk)

TABLE 2 The effects of sex, age and their interaction on proliferative changes to liver (a), deposition of lipofuscin (b) and proliferative changes to kidney (c). Parameter estimates and *z*-statistics of generalized mixed models are presented

Organ impairment	Factor	Estimate	± *SE*	*z*-value	*p*
Lipofuscin—liver	Intercept	3.099	±0.263	11.78	<0.001
	Sex (males)	−0.045	±0.259	−0.17	0.862
	Age (young)	0.071	±0.045	1.57	0.115
	Sex × Age	−0.195	±0.299	−0.65	0.514
Proliferative changes—kidney	Intercept	0.338	±0.347	0.97	0.331
	Sex (males)	−0.481	±0.281	−1.71	0.087
	Age (young)	−0.637	±0.353	−1.81	0.071
	Sex × Age	0.551	±0.474	1.16	0.247
Proliferative changes—liver	Intercept	0.761	±0.242	3.14	0.002
	Sex (males)	−0.110	±0.218	−0.50	0.615
	Age (young)	−0.240	±0.225	1.07	0.287
	Sex × Age	0.449	±0.310	1.45	0.147

We detected no differences between the sexes in measures of functional aging (lipofuscin deposition and proliferative changes in liver and kidney).

The demography of male-biased mortality in natural and experimental annual killifish populations is well explained in terms of sexual selection. In the wild, there is evidence that males suffer elevated predation. Annual killifish are highly sexually dichromatic, with brightly coloured males and dull females (Sedláček et al., 2014), and visually hunting birds, such as herons and kingfishers, are the main predators of annual killifish (Haas, 1976; Keppeler et al., 2016; Reichard & Polačik, 2019). The mortality cost of showy sexually selected traits is a well-recognized source of intersexual differences in life span (Lemaître et al., 2020; Promislow et al., 1992; Székely et al., 2014) and our data suggest male-biased mortality in wild populations of all four species.

Using data from captive breeding, we then excluded predation (and predation risk). We predicted that sex differences mortality and life span would disappear in both laboratory treatments, if predation is the exclusive source of male-biased mortality. If social stress from male–male competition for access to reproduction (rather than predation, or in addition to it) elevates male mortality, we predicted the persistence of male-biased mortality in the social housing treatment but no sex differences in singly housed treatment. The absence of predation eliminated the sex bias in mortality in two species, but not in the other two. This interspecific variation in the role of social interactions in the sex bias in life span tightly covaries with the level of male aggressiveness. Male aggression is markedly highest in *N. orthonotus*, followed by *N. furzeri* and *N. kadleci* and the lowest in *N. pienaari* (Genade, 2005; Polačik & Reichard, 2011; Wildekamp, 2004). Hence, male–male competition for mating opportunities significantly contributed to elevated male mortality in species which were previously recognized as more aggressive. We also directly observed male combat-related injuries in five *N. orthonotus* and three *N. furzeri* males that died in the social group treatment. Sublethal conditions, such as persistent stress (Keller et al., 1992), possibly mediated by elevated levels of corticosteroids (Foo et al., 2017), are frequently associated with increased mortality (Moore & Wilson, 2002), but hormonal profiles were not measured in our study.

Unexpectedly, we detected no functional characteristics underlying a higher male baseline mortality in our study, despite using measures of functional aging that were previously found to be suitable biomarkers of functional decline as they varied predictably with age and among killifish species and populations (Baumgart et al., 2015; Blažek et al., 2017; Terzibasi-Tozzini et al., 2013). This outcome is comparable to the well-described male–female health-survival paradox in humans, where woman outlive men despite experiencing greater levels of functional problems at older age (reviewed in Gordon et al., 2017). This paradox may be more widespread, but not well recognized, across animals (e.g. *Drosophila melanogaster*: Regan et al., 2016). In our study, however, female functional declines were equal, and not higher, that those detected in males. We also failed to detect a re-appearance of male bias in mortality under challenging environmental conditions generated in the laboratory. We acknowledge that our measures of functional ageing may be considered too strong and other markers would be more sensitive and more powerful to detect sex differences in functional ageing. For example, male *N. furzeri* have consistently shorter telomeres than females (Reichard et al., 2022).

One species (*N. kadleci*) exhibited equal adult sex ratios in the wild and a male-biased sex ratio at sexual maturity in captivity, consistently across populations and cohorts. Sowersby et al. (2020) hypothesized that sex ratios can evolve extremely rapidly in killifish, demonstrating large interspecific differences among adult sex ratios across 15 annual and non-annual killifish species raised in the laboratory. We propose that a male-biased sex ratio in *N. kadleci* might have evolved as a compensatory mechanism to mitigate male-biased mortality in natural populations. Some wild populations in our dataset presented extremely female-skewed sex ratios (e.g. in one natural population we collected 44 females and only one male), and the production of male-biased progeny would be adaptive in such populations in accordance with Fisher's principle (Fisher, 1930). Ongoing cytogenetic research will characterize the nature of this potential compensatory mechanism.

Despite an enormous research effort, a comprehensive causal understanding of sex differences in life span and aging remains elusive, probably because it comprises a series of complex underlying sources. Mammals are arguably the best studied vertebrate taxon with respect to aging. A recent comparative study that combined data from wild populations of 101 mammalian species demonstrated that adult females lived on average 19% longer than conspecific adult males but without finding any consistent intersexual differences in aging rates (Lemaître et al., 2020), in line with our experimental results with killifishes. The fact that heterogametic-sex disadvantage is much stronger in male heterogametic systems (21% longer life span of homogametic females) than female heterogametic systems (7% longer life span of homogametic males) (Xirocostas et al., 2020) highlights the importance of reproductive roles and mating systems in shaping intersexual life span differences. Here, we have demonstrated how sexual selection (especially sexually dimorphic behaviour and coloration, and the strength of male–male competitive interactions over access to mating) affects sex differences in life span and aging through multiple processes even within an ecologically and evolutionary discrete lineage, and that these effects are strongly moderated by social and environmental conditions.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

M.R. conceived and designed the study, conducted the statistical analyses and drafted the manuscript; M.R., R.B. and M.P. collected data on wild populations; R.B. completed the experiment with captive fish, with the assistance of M.P.; J.Z. collected data from fluctuating temperature; A.C. and R.B. performed the histological analysis. All authors contributed to the final text.

DATA AVAILABILITY STATEMENT

Primary data are deposited on the Figshare repository https://doi.org/10.6084/m9.figshare.12752648.v2 (Reichard, 2020).

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