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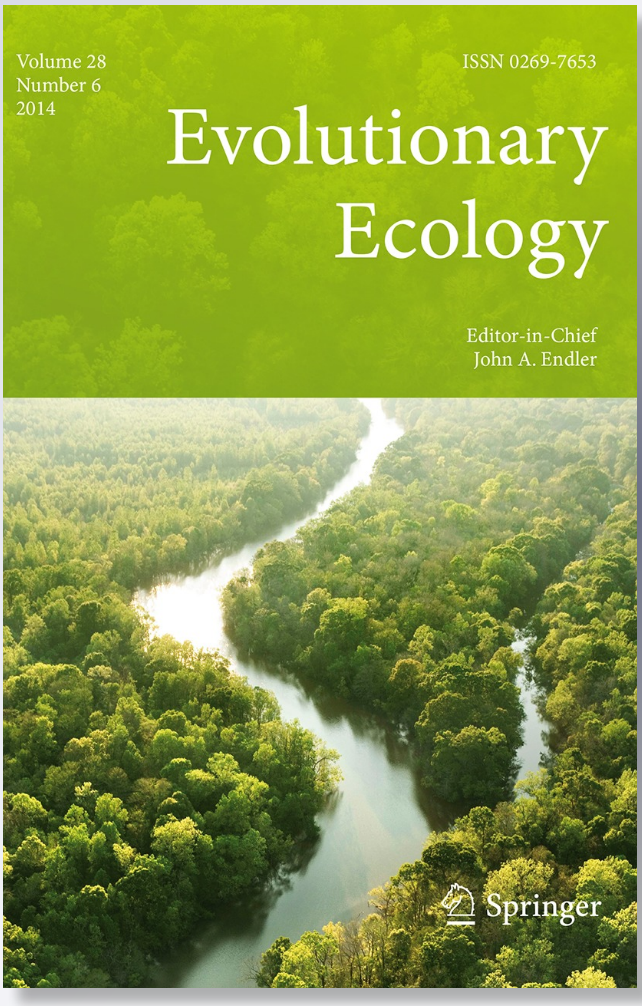

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ORIGINAL PAPER

Female bias in the adult sex ratio of African annual fishes: interspecific differences, seasonal trends and environmental predictors

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Abstract Many populations have consistently biased adult sex ratios with important demographic and evolutionary consequences. However, geographical variation, the mechanisms, temporal dynamics and predictors of biased sex ratios are notoriously difficult to explain. We studied 334 wild populations of four species of African annual fish (*Nothobranchius furzeri*, *N. kadleci*, *N. orthonotus*, *N. rachovii*) across their ranges to compare their adult sex ratio, its seasonal dynamics, interpopulation variation and environmental predictors. *Nothobranchius* populations comprise a single age cohort and inhabit discrete isolated pools, with wide-ranging environmental conditions (habitat size, water turbidity, structural complexity, predators), making them ideal to study adult sex ratio variation. In captivity adult sex ratios were equal. In natural populations, adult sex ratios were biased 1:2 toward females in three study species while *N. kadleci* had sex ratios at unity. There was a decline in the proportion of males with age in one species, but not in the other species, implying most severe male mortality early after maturation, declining later perhaps with a decrease in male abundance. In general, the populations at vegetated sites had relatively more males than populations at sites with turbid water and little vegetation. Selective avian predation on brightly coloured male fish likely contributed to female dominance and vegetation cover may have protected males from birds. In addition, an aquatic predator, a large belastomid hemipteran, decreased the proportion of males in populations, possibly due to greater male activity rather than conspicuous colouration. Alternative explanations for a sex ratio bias, stemming from male–male contests for matings, are discussed. We conclude that the effect of environmental conditions on adult

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sex ratio varies dramatically even in closely related and ecologically similar sympatric species. Therefore, difficulties in explaining the ecological predictors of sex ratio biases are likely due to high stochasticity rather than limited sample size.

Keywords Killifish · Tertiary sex ratio · Predator bias · Temporary savanna pools · Demographic consequences

Introduction

Adult sex ratio, the proportion of sexually mature males and females in a population, is a key demographic parameter. Many populations have consistently biased sex ratios, with fundamental demographic and evolutionary consequences (Clutton-Brock et al. 1997; Hardy 2002; Field et al. 2012; Székely et al. 2014a). The primary sex ratio concerns the proportion of each sex at fertilization. The secondary sex ratio describes the proportion of the sexes at birth, while the tertiary sex ratio (termed 'adult sex ratio' henceforth) is the proportion of the sexes during sexual maturity. A bias in sex ratio may arise at any developmental stage (Hardy 2002). Biased sex ratios may be adaptive and maternally induced (fig wasps: Raja et al. 2008), caused by parasite infection (*Wolbachia*: Hurst et al. 1999), or by environmental interference with sex determining system (natural: Römer and Beisenherz 1996; human-induced: Jobling et al. 1998) and consistently biased from the onset of sexual maturity. However, unequal adult sex ratios may also be caused by sex-biased mortality (Promislow et al. 1992; Reale et al. 1996; Lodé et al. 2004) and may be temporally dynamic over the lifetime of an age cohort (Forsgren et al. 2004).

Both males and females may be the rarer sex as adults (Székely et al. 2014a). Females may suffer increased mortality rates compared to males following energetically expensive reproduction (Clutton-Brock et al. 1989) or due to intensive male harassment (Reale et al. 1996). Alternatively, they may be the rarer sex when sex roles are reversed (Kosztolányi et al. 2011). More often, however, populations are female-biased (Boukal et al. 2008). Increased male mortality is typically associated with costs of mate searching and courtship (Lodé et al. 2004), advertisement of conspicuous ornaments to attract females (Promislow et al. 1992) or male–male competition for territories and mating opportunities (Le Boeuf 1974). It is generally recognized that an increase in female bias with cohort age comes from selective male mortality, but the mechanisms, temporal dynamics, and predictors of sex-biased mortality are often opaque (Hardy 2002; Székely et al. 2014a). Importantly, a recent meta-analytical approach concluded that reports on biased adult sex ratios may often be false due to small sample size or sampling bias (Wehi et al. 2011). To this end, a comparative approach can illuminate factors responsible for selective male mortality across populations and among closely related species (McKellar et al. 2009; Wehi et al. 2011).

Here, we use multiple populations of four species of African annual fish to examine consistent biases in adult sex ratio, and the potential mechanisms producing such bias. *Nothobranchius* are small teleost fish with striking sexual dichromatism (Fig. S1). Females are light brown with ornamentation limited to small spots in some species and no ornamentation in others. In contrast, males possess highly conspicuous nuptial colouration (Haas 1976a; Sedláček et al. 2014), which is species-specific (Wildekamp 2004). Sex determination is genotypic (Ewulonu et al. 1985; Valenzano et al. 2009), implying a 1:1

primary sex ratio (Hamilton 1967). *Nothobranchius* display no alternative mating tactics and sexes can be readily distinguished in adults (Reichard et al. 2009; Polačik et al. 2014a). *Nothobranchius* inhabit isolated rain-fed pools in the African savannah that are subject to annual desiccation. Additionally, the presence of adults effectively halts the development of eggs (Inglima et al. 1981), making generations strictly non-overlapping (Polačik et al. 2014a). During the rainy season, fish hatch from desiccation-resistant eggs and reach sexual maturity in 3–6 weeks. Hatching is synchronous and each pool consists of a single age cohort (Polačik et al. 2011). Pools vary in size, heterogeneity (such as the presence of vegetation) and community composition, including the presence of potential predators (Reichard et al. 2009; Reichard 2010). After reaching maturity, *Nothobranchius* reproduce daily, with females laying 5–50 eggs each day in separate spawning events. Males compete aggressively for access to females (Haas 1976b). Females are attracted by males (Haas 1976a), implying that males need to be seen by females for successful reproduction. Therefore, male colouration may be subject to sexual selection via sensory bias (Rodd et al. 2002), which can potentially be exploited by predators (e.g. Rosenthal et al. 2001).

We used an exceptionally large dataset of adult sex ratios from 334 wild populations of 4 *Nothobranchius* species from southern and central Mozambique to test: (1) whether there was a consistent sex ratio bias and whether it varied among the four species; (2) whether there was a temporal trend in the adult sex ratio, implying an ongoing male-biased mortality over the lifetime of an age cohort; and (3) what habitat characteristics were associated with adult sex ratio bias. We finally used a large dataset of sex ratios at sexual maturation for wild-derived captive populations to compare adult sex ratios in a protected and unprotected environment. The advantage of using *Nothobranchius* fish to study adult sex ratio bias is their repeated occurrence in strictly isolated populations comprising a single age cohort and non-overlapping generations, and across a range of environmental conditions. *Nothobranchius* sampling is unbiased towards a particular sex, the sex determination system is genotypic and several species co-occur in the same pools.

Materials and methods

Species

The four species of *Nothobranchius* included in our study are largely sympatric. *Nothobranchius furzeri* Jubb occurs in southern Mozambique between the Incomati and Save river basins, with a single locality known in south-eastern Zimbabwe (Jubb 1971; Bartáková et al. 2013). *Nothobranchius orthonotus* (Peters) is distributed across southern and central Mozambique, with a marginal distribution in KwaZulu-Natal in the south and potential occurrence in southern Malawi (Wildekamp 2004; Dorn et al. 2011). *Nothobranchius kadleci* Reichard is an ecological vicariant of *N. furzeri*, distributed between the Save and Zambezi rivers (Reichard 2010). Finally, *Nothobranchius rachovii* Ahl is largely sympatric with *N. orthonotus* across its entire range (Wildekamp 2004). Recently, the species has been divided into three separate species (*N. rachovii* Ahl sensu stricto, *Nothobranchius pienaari* Shidlovskiy, Watters, Wildekamp and *Nothobranchius krysanovi* Shidlovskiy, Watters, Wildekamp) based on male colouration, geographic distribution and cytogenetic differences (Shidlovskiy et al. 2010). Given the vicariant character of these three species, their shared ecology and the fact that the distinction has been made after our data from 2008 to 2010 had already been already collected, we pool them in *N. rachovii* sensu lato in this paper. Note that most (estimate of 51 out of 62 populations on the basis of geographical distribution) *N. rachovii* sensu lato samples belong to populations of *N. pienaari*, which has the most extensive distribution, with the other two species occurring in isolated areas (Shidlovskiy et al. 2010).

Data collection

The study was conducted in southern and central Mozambique (from the basin of the Incomati to the basin of the Zambezi river), including virtually the entire ranges of the four study species (Wildekamp 2004; Dorn et al. 2011). Estimates of adult sex ratios in wild populations with corresponding habitat variables come from 8 field expeditions conducted between 2008 and 2012. Fish were collected using a dip net with a triangular metal frame (45 × 45 cm, mesh size 5 mm) on a long (1.5 m) wooden pole. Typically, 15–40 hauls were performed at each site but more hauls were performed when fish density was low. Fewer hauls were taken if the habitat was too small to accommodate 15 hauls. At some sites, a beach Seine net (length 2.7 m, depth 0.7 m, mesh size 4 mm) was used in addition or as an alternative to the dip net. The mesh size used retained adult *Nothobranchius* unselectively and there was no sex bias in the probability of capture. A potential sex bias in capture was estimated on the basis of repeated removal sampling (n = 8 populations; 3,984 fish) and capture-mark-recapture sampling (a single population, 901 fish). The first removal sample (which corresponded to an estimate of sex ratio in most populations) always described the sex ratio correctly; there was no difference between the estimates from the first sample and the overall pooled estimate, Goodness-of-Fit test, all $P > 0.05$).

In the years 2011 and 2012, pool area (estimated to the nearest 10 m^2), water turbidity (ordinal scale: transparent: bottom visible; turbid: visibility 1–10 cm; very turbid: visibility <1 cm), the presence of predatory lungfish (*Protopterus annectens* (Owen), present/absent) and predatory hemipterans (Belostomatidae; present/absent) and vegetation cover (separately for *Nymphaea* sp., submergent aquatic vegetation and grassy vegetation within the pool, including its margins, all visually estimated as percentage cover) were recorded at each site as potential predictors of the adult sex ratio. All fish were sexed and identified to species. Most fish were returned to the pools but in some cases a subsample was taken to the laboratory for further research. In the few cases when juvenile fish were recorded along with young adults, estimates of sex ratio were not made as juveniles cannot be reliably sexed without dissection.

A high proportion of sites were resampled among years, but full replication of all sampling sites was not possible due to the inclusion of new sites in some years, inaccessibility of certain sites in particular years and temporal variability in the presence of water and fish among years. More remote regions were not visited annually. In 2010 and 2012, sites were sampled early and late in the season, during two separate expeditions (10 February to 4 March and 5–16 April 2010, 26 February to 16 March and 22 May to 6 June 2012). Given the estimated hatching date of *Nothobranchius* between late December and mid January each year (Polačik et al. 2011), fish were 1–2 months old during the early season sampling and 3–4 months (in 2010) or 5–6 months (in 2012) old during late season sampling. In other years, fish were always sampled early in the season (8–23 February 2008, 6–26 February 2009, 24 February to 13 March 2011).

Captive assays

All data on captive fish are based on populations we imported between 2008 and 2012. Laboratory culture followed established protocols (Genade 2005; Blažek et al. 2013).

In short, oviposited eggs were stored in moist peat moss substrate in a temperature controlled incubator at 22 °C. Embryos were hatched in 4 L tanks with well-oxygenated tapwater (15–17 °C). Water temperature was gradually increased to 24–26 °C. Fish were raised in large groups of individuals (30–120) from the same cohort (hatching event) and population. Fish were fed ad libitum with brine shrimp nauplii 2–3 times each day and weaned onto live and frozen bloodworm at an age of 7–14 days. Fish were kept under 14 h:10 h light:dark regime in aged tap water (conductivity 550 μS cm^{-2}), with 30–50 % water exchanges 1–3 times each week. At sexual maturity the sex of each individual was scored based on colouration. Captive assays were completed in our fish facility at the Institute of Vertebrate Biology in Brno, Czech Republic. In two cases, adult sex ratio estimates were based on a cohort hatched and raised at Portland State University, Oregon, USA where conditions were comparable except for water conductivity (100 μS cm^{-2}) and fish were housed in smaller groups (5–10 individuals).

Data analyses

For all statistical analyses, we only used data from populations where the total number of individuals was larger than 5 to avoid spurious outcomes due to small sample size. The sample size effect was accounted for by using raw binomial data rather than percentages (Wilson and Hardy 2002). For discussion and graphical display, sex ratios are expressed as proportion of males. To obtain overall estimates of adult sex ratio for wild populations of individual species and to compare sex ratios among species, we fitted Generalized Linear Mixed Model (GLMM) with binomial error structure (male to female ratio) and log-link function in the *lmer* package in the R statistical environment (Pinheiro and Bates 2000; R Development Core Team 2005), where Species were treated as fixed factor and Year and Site as random factors. We also fitted another GLMM with Year as a fixed rather than random effect (including Year by Species interaction as the fixed effect) to formally evaluate whether there was significant inter-annual variability in sex ratio.

For testing seasonal change in sex ratio, the GLMM with binomial error structure and log-link function was fitted where Season (levels Early and Late) was a fixed factor and Year and Site were random factors. For this analysis, only data from 2010 and 2012 were used given repeated sampling during the wet season in these years. Populations of *N. kadleci* were not analyzed for seasonal change due to their small sample size. Finally, we tested the effect of predictor variables on sex ratio bias. Predictors included presence of predatory lungfish and hemipterans (binary variables), size of pool (ln transformed), turbidity (ordinal scale: clear, intermediate, or turbid water sensu Reichard et al. (2009)) and extent of *Nymphaea* sp., aquatic and grassy vegetation (0–100 % cover for each vegetation type). Given strong positive correlations between vegetation types and their negative correlations with water turbidity, we collapsed the data matrix using principal component analysis (PCA) to avoid predictors redundancy. Two axes had eigenvalues >1. The first (PC1, eigenvalue = 2.07) explained 51.8 % of variation and was strongly negatively associated with turbidity (−0.81) and positively associated with *Nymphaea* sp. (0.76) and aquatic vegetation (0.73). The second (PC2, eigenvalue = 1.17) explained 29.3 % of variation and was strongly positively associated with grassy vegetation (0.74).

Given species-specific habitat use, trophic ecology, male colouration and sex-ratio bias, we treated each species separately in this analysis. We fitted GLMMs as for the overall analysis, though the number of observations was limited to the years 2011 and 2012 due to the absence of reliable predictor data from the years 2008, 2009 and 2010. First, we fitted a full model including all predictors as fixed effects and Year and Site as random effects.

We then used the *MuMIn* package (Bartoń 2012) to evaluate the fit of all candidate models using an AICc (Akaike Information Criterion corrected for a small sample size) based approach. All potential models (i.e. all combinations of fixed factors; random effects were always retained) were ranked according to their AICc and their ΔAIC (difference between AICc of the best model and compared model) was calculated. A subset of models that had comparable fits to the data ($\Delta\text{AICc} < 2$) was used to produce model-averaged parameter estimates (Burnham and Anderson 2002). We further report the relative importance of the predictors for each species. The best model was always compared to the null model (i.e. only random effects considered) using a log-likelihood ratio test to examine whether the models with predictors explained significantly more variability than the null models. Sample size for each analysis and species are given in Table 1.

Results

Inter-specific differences

Sex ratio was significantly female-biased in all species except for *N. kadleci* (Fig. 1); the 95 % confidence intervals for sex ratio in *N. furzeri*, *N. orthonotus* and *N. rachovii* did not overlap with a value of 0.5 (Table 2). The equal sex ratio in *N. kadleci* was not an artifact of geographical or environmental conditions in the range of *N. kadleci* because a subset of *N. orthonotus* and *N. rachovii* populations that were sympatric with *N. kadleci* still had significantly female-biased sex ratios (*N. orthonotus*: mean 0.38, 95 % confidence interval 0.30–0.47, $n = 19$; *N. rachovii*: mean 0.33, 95 % CI 0.23–0.46, $N = 4$; GLMM: $P = 0.001$), and these estimates did not differ to those obtained from the entire dataset for the two species (i.e. confidence intervals broadly overlap; see Table 2). The effect of the random factor Year (variance 0.00008) was negligible compared to the random factor Site (variance 0.2065). High inter-annual congruence in sex ratios was confirmed when Year was modelled as a fixed factor (GLMM, Year: $P = 0.851$, Year by Species interaction: $P = 0.345$).

Seasonal trends

There was a significant decline in the proportion of males in populations of *N. furzeri* between the early and late seasonal sampling (GLMM with binomial error, $z = -5.11$, $n = 60$, $P < 0.001$; mean (95 % confidence interval) proportion of males 0.371 (0.338–0.405) for early sampling and 0.212 (0.161–0.274) for late sampling) (Fig. 2). No decline was detected in *N. orthonotus* ($z = 1.07$, $n = 56$, $P = 0.273$) and *N. rachovii* ($z = -0.42$, $n = 32$, $P = 0.677$). We acknowledge, however, that limiting our dataset to a paired design (i.e. including only pools sampled during both field trips in respective years), there was no significant change in the sex ratio between early and late stages of the pool's existence neither in *N. furzeri* (paired t test on square-root transformed data: $t_{11} = 1.05$, $P = 0.317$).

Habitat predictors of sex ratio bias

The relationship between predictor variables and variability in sex ratios among populations varied among species. In *N. furzeri*, relatively more males were found at sites with

Table 1 The number of populations and individuals (in parenthesis) used in analyses and the number of unique populations

	Overall analysis	Habitat predictors	Seasonal trends: early season	Seasonal trends: late season	Unique populations
N. furzeri	128 (9,291)	51 (5,534)	37 (3,329)	23 (2,429)	60
N. orthonotus	115 (2,781)	58 (1,564)	36 (1,001)	20 (445)	82
N. kadleci	30 (852)	16 (425)	10 (243)	8 (251)	19
N. rachovii	61 (1,282)	39 (1,026)	18 (397)	14 (379)	42

Only populations with more than 5 fish were considered in analyses and are included in the table. Note that populations were sampled in up to 5 different years and thus contributing to 1–5 population estimates

abundant aquatic vegetation, *Nymphaea* and clearer water (positive association with PC1, Table 3a). In *N. rachovii*, relatively more males were collected in pools that were larger, lacked predatory hemipterans and had more grassy vegetation (positive association with PC2, Table 3b). In *N. kadleci*, a null model fitted the data best (ΔAIC to the best model with a predictor factor $= 2.66$), implying no effect of predictors on sex ratio variation in *N. kadleci*. In *N. orthonotus* predictors also had no effect on variation in sex ratio as the null model received equal support to the best models with predictors (ΔAIC $= 1.03$; log-likelihood test, $P > 0.05$).

Captive populations

In most populations and hatching events ($N = 34$), there was no sex ratio bias in wild-derived captive populations when fish reached sexual maturity (Table 4, $P > 0.05$), except for a single case when males were more abundant (exact binomial test, $P = 0.008$). An equal sex ratio was also confirmed for species-specific and overall sums across populations and hatching events (exact binomial test, all $P > 0.05$).

Discussion

It is widely recognized that adult (tertiary) sex ratios in natural populations can vary temporally and spatially across a species' range but it has proven notoriously difficult to identify the causes of this variation (McKellar et al. 2009). We used an exceptionally large dataset and compared the adult sex ratios of 334 populations to examine interspecific differences in the adult sex ratio, its seasonal dynamics and environmental correlates. Our data demonstrated that wild populations of *Nothobranchius* are typically female-biased but with a notable exception of one species, *N. kadleci*. Given that captive populations had equal sex ratios at sexual maturity, sex ratio bias appears to stem from male mortality and involves a strong environmental component. There was some seasonal decline in the proportion of males with age in *N. furzeri*, but the strongest male mortality likely occurs early in life as no intensification in female bias was observed in other species (where sample size was lower). Notably, populations from vegetated sites had relatively more males than populations at sites without vegetation and with turbid water, though the effect was apparent only in two of the three species with female-biased adult sex ratios. Habitat size and the presence of predatory hemipterans also explained part of the sex-bias.

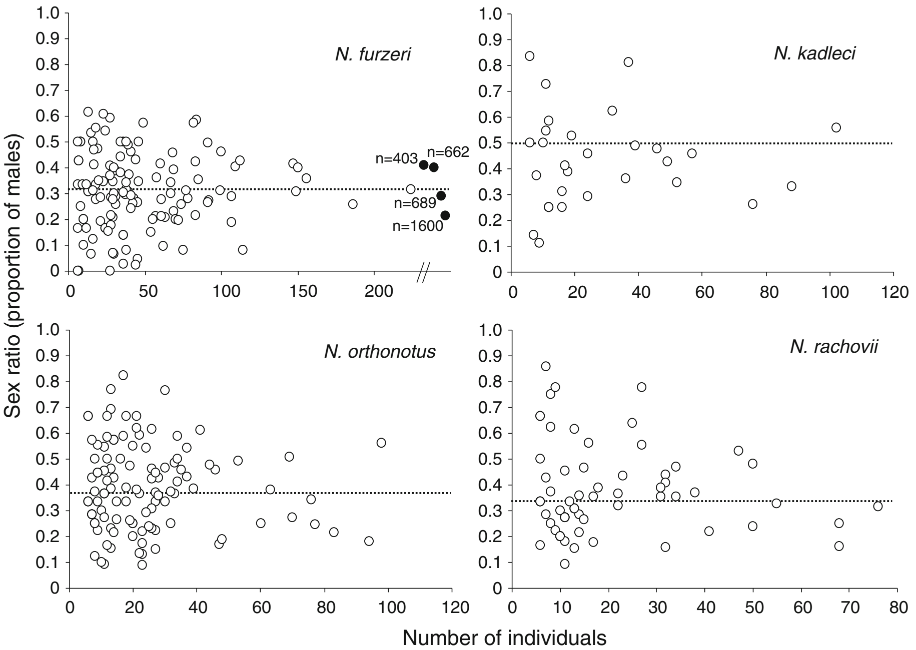

Fig. 1 Adult sex ratios (proportion of males) in wild populations of *Nothobranchius* plotted against sample size for each estimate. Mean values estimated from GLMM analyses are indicated by a dotted line. *Closed symbols* represent population estimates with unusually large sample size, offset for graphical display

Table 2 Estimates of adult sex ratio in wild populations of four *Nothobranchius* species in the years 2008–2012

Species	Model estimates			Normalized estimates	
	Mean (SE)	*z*	*P*	Mean	95 % CI
N. furzeri	−0.751 (0.061)	−12.27	<0.001	0.321	0.295–0.347
N. orthonotus	−0.489 (0.062)	−7.84	<0.001	0.380	0.352–0.409
N. kadleci	−0.020 (0.114)	−0.18	0.859	0.495	0.439–0.551
N. rachovii	−0.677 (0.084)	−8.01	<0.001	0.337	0.301–0.375

Estimates of the Generalized Mixed Models with binomial error structure (with standard error in parentheses), corresponding z-values and statistical significance are given along with the estimates (mean and 95 % confidence intervals) recalculated to normal distribution (0: only females, 0.5 equal sex ratio, 1: only males)

Variation in sex ratio biases among related species

In our study, the mean proportion of males in wild populations was one-third in three species, but the forth species (*N. kadleci*) had an equal adult sex ratio. The within-species variation across populations was large (Fig. 1), but adult sex ratios were congruent among years. The departure of a single species from a general pattern is difficult to explain. Male colouration varies among species, but *N. kadleci* possesses the brightest male colouration among the study species, at least to the human eye; male *N. kadleci* are bright red over their

Fig. 2 Mean adult sex ratio in populations of *N. furzeri* sampled early and late in the season. Whiskers represent 95 % confidence intervals

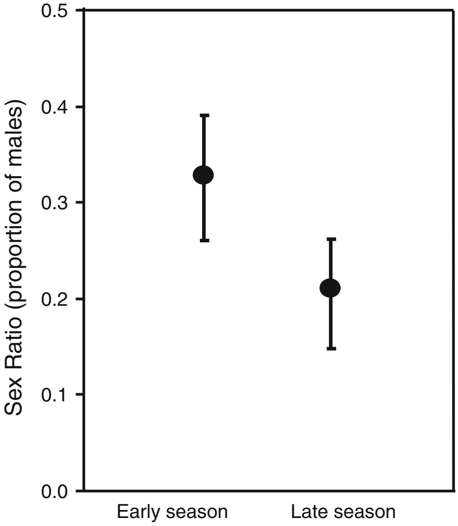

Table 3 Results of generalized mixed model analysis of the effect of habitat variables on sex ratio in (a) *N. furzeri* and (b) *N. rachovii*

Variable	Relative importance	Estimate	SE	*z*	*P*
(a) *N. furzeri*					
PC1	1.00	0.207	0.058	3.59	0.0003
Hemipterans	0.34	−0.156	0.143	1.09	0.275
(b) *N. rachovii*					
Pool size	1.00	0.126	0.044	2.89	0.004
Hemipterans	1.00	−0.490	0.191	2.57	0.010
PC2	1.00	0.180	0.075	2.41	0.016
Lungfish	0.42	−0.343	0.220	1.56	0.119

The relative importance of each predictor, model-averaged coefficient estimates for predictors and their adjusted standard errors (*Mirounga augustirostris*), test characteristic (*z*) and statistical significance (*P*) are shown for a composite model based on two models which had equal support based on AICc (i.e. within 2 units of AICs compared to the best model)

entire body and fins (Fig. S1). Thus if male colouration is responsible for selective mortality (for specific discussion, see below), *N. kadleci* males should be the most conspicuous to predators and consequently suffer the greatest mortality. The level of male aggression (another potential source of male mortality) also varies among species, though *N. kadleci* together with *N. furzeri* is intermediate between the most aggressive *N. orthonotus* and the relatively benign *N. rachovii* (Genade 2005; Genade et al. 2005; Polačik and Reichard 2011; R. Blažek and M. Polačik, unpublished data). *N. kadleci* and *N. furzeri* also have similar ecological requirements (Polačik et al. 2014b). The difference in sex ratio among species cannot be attributed to specific habitat conditions at the sites containing *N. kadleci*, as a subsample of *N. orthonotus* and *N. rachovii* populations sympatric with *N. kadleci*

Table 4 The number of males and females at sexual maturity across 19 captive populations imported from Mozambique and kept in the laboratory for 1–3 generations prior to sex ratio estimates

Species and population code	Geographic origin	Males	Females	Sex ratio
N. furzeri MZCS 121	Limpopo	77	74	0.43–0.59
N. furzeri MZCS 121	Limpopo	23	31	0.29–0.57
N. furzeri MZCS 121	Limpopo	16	11	0.39–0.78
N. furzeri MZCS 002	Limpopo	20	32	0.25–0.53
N. furzeri MZCS 002	Limpopo	57	46	0.45–0.65
N. furzeri MZCS 001	Limpopo	19	12	0.42–0.78
N. furzeri MZCS 122	Limpopo	17	20	0.30–0.63
N. furzeri MZCS 304	Limpopo	14	9	0.39–0.80
N. furzeri MZCS 222	Chefu	80	88	0.40–0.56
N. furzeri MZCS 222	Chefu	14	14	0.31–0.69
N. furzeri MZCS 222	Chefu	37	34	0.40–0.64
N. furzeri MZCS 222	Chefu	58	32	0.54–0.74 *
N. furzeri MZCS 222 (USA)	Chefu	48	32	0.43–0.64
N. furzeri MZCS 222 (USA)	Chefu	24	15	0.45–0.77
N. furzeri MZCS 024	Chefu	4	9	0.09–0.61
N. furzeri MZCS 024	Chefu	21	17	0.38–0.71
N. furzeri MZCS 034	Chefu	86	70	0.47–0.63
N. furzeri MZCS 414	Chefu	26	32	0.32–0.59
N. furzeri MZCS 422	Chefu	15	14	0.33–0.71
N. orthonotus MZCS 002	Limpopo	83	74	0.45–0.61
N. orthonotus MZCS 002	Limpopo	36	32	0.41–0.65
N. orthonotus MZCS 528	Beira	78	80	0.41–0.57
N. orthonotus MZCS 528	Beira	36	43	0.34–0.57
N. kadleci MZCS 091	Gorongosa	71	71	0.42–0.59
N. kadleci MZCS 091	Gorongosa	19	25	0.28–0.59
N. kadleci MZCS 108	Save	14	13	0.32–0.71
N. kadleci MZCS 109	Save	6	4	0.26–0.88
N. kadleci MZCS 091	Save	11	10	0.30–0.74
N. kadleci MZCS 430	Nhamatanda	81	73	0.44–0.61
N. kadleci MZCS 430	Nhamatanda	12	12	0.29–0.71
N. kadleci MZCS 367	Nhamatanda	18	20	0.31–0.64
N. kadleci MZCS 512	Buzi	66	47	0.49–0.68
N. rachovii MZCS 505	Limpopo	115	106	0.45–0.59
N. rachovii MZCS 514	Beira	106	117	0.41–0.54

95 % confidence intervals of the sex ratio (proportion of males) estimate, calculated using exact binomial tests, are shown. A significant deviation from 1:1 adult sex ratio is indicated with an asterisk

displayed the same female-biased adult sex ratio as that observed overall for these species. Thus the distinct outcome for *N. kadleci* remains unexplained. While this finding contrasts with a comparable study that reported broad congruence in adult sex ratios among 6 species of ecologically similar tree wetas (*Hemideina* spp.), ensiferan crickets with clear sexual dimorphism (Wehi et al. 2011), an extensive review of adult sex ratios in birds demonstrated that several species defied the general pattern of sex ratio bias otherwise typical for their ecological guild (Székely et al. 2014b).

Seasonal dynamics in adult sex ratios

Adult sex ratios vary over the age of a cohort (Hardy 2002). The dynamics are often linked to reproductive effort and may be either intrinsic or extrinsic. Intrinsic mortality results from decreased physiological condition as a consequence of male mating effort. Extrinsic mortality occurs when predators directly target male sexual advertisement (Rosenthal et al. 2001; Lodé et al. 2004), or males suffer mortality fighting for access to females (Le Boeuf 1974). While either cause will result in an increasingly female biased sex ratio over the lifetime of the cohort, extrinsic mortality would be expected to have an impact much earlier in the mating season than intrinsic mortality. We suspected that the source of male mortality was extrinsic and thus predicted that the sex ratio would become female biased soon after sexual maturity (during our first sampling) and increase over time (detected as seasonal increase in sex ratio bias). Support for this prediction of equivocal.

Nothobranchius hatch synchronously at the peak of the rainy season, typically during intensive monsoon precipitation in the first half of January (Polačik et al. 2011; Terzibasi Tozzini et al. 2013). For seasonal comparison, our first sampling occurred only 2–5 weeks after the fish reached sexual maturity. In contrast, during the second sampling the fish would have been sexually mature for 8–18 weeks and the population thus prone to much longer period of male-biased mortality. While we demonstrated a significant decline in the proportion of males in *N. furzeri* (from a mean male proportion of 0.37 (i.e. 1 male: 2.7 females) to 0.21 (1 male: 4.7 females), no significant decline was detected in an alternative statistical analysis (using only paired matched samples) and in other study species. This result suggests that male-bias in mortality is high initially and decreases with a decline in male density. It is noteworthy that the same bias in adult sex ratios (one-third of males in a population) is reported as relatively stable in four populations of Trinidadian guppies (Arendt et al. 2014). In the laboratory, there is no apparent sex difference in mortality rate in *Nothobranchius* (e.g. Lucas-Sánchez et al. 2011; Terzibasi Tozzini et al. 2013), suggesting that selective male disappearance from wild populations must have a strong environmental component. This finding is important because the role of elevated male mortality in biased adult sex ratios has only recently been established (Arendt et al. 2014; Székely et al. 2014b) and direct comparison of adult sex ratios between wild and captive populations is rarely accomplished (Harper 2010).

Mechanisms producing sex ratio bias

Selective predation of visually and behaviourally conspicuous males (Haas 1976a; Promislow et al. 1992) or male–male aggressive competition for mating opportunities (Le Boeuf 1974; Genade et al. 2005) are the most likely immediate sources of male mortality across taxa. In our analysis, lungfish and predatory hemipterans were included as potential predators of *Nothobranchius* (Greenwood 1986; Tobler et al. 2007). While lungfish had only a marginal impact on the proportion of *Nothobranchius* males, the effect of predatory hemipterans was stronger (Table 3). We have witnessed many cases when large predatory hemipterans fed on *Nothobranchius* after capture in nets and our observations in captivity confirm that predatory hemipterans are potent predators of fish. A single hemipteran collected from a pool with *Nothobranchius* consumed up to 3 fish of a comparable size to adult *Nothobranchius* in 1 week, using sit-and-wait tactics (R. Blažek, personal observation). Their predation on fishes appears common and often size- or sex-biased. For example in cave mollies (*Poecilia mexicana*), belostomatid predation is biased towards larger fish (Tobler et al. 2007) and is additionally male-biased, due to the higher activity of males during mate searching and courtship (Tobler et al. 2008). Lungfish, likewise, do not base their foraging on visual cues (Greenwood 1986) and we found only a single lungfish with *Nothobranchius* remains in its stomach (at a site with a high density of adult *Nothobranchius*), out of a sample of 16 adult lungfish dissected across several sites. The diet of lungfish typically consisted of molluscs and crabs rather than fish (M. Polačik, unpublished data). Consequently, male *Nothobranchius* may be selectively targeted by aquatic predators (mainly belostomatids) due to their more active behaviour (Polačik and Reichard 2011) rather than their colouration.

Vegetation cover was the strongest predictor of sex ratio bias, with a positive association between the amount of vegetation cover and the proportion of males at a site. A possible explanation is that that greater vegetation cover may have provided refuge to male *Nothobranchius* from bird predation compared to sites without vegetation. Predation by birds, such as herons and kingfishers is likely to be a major source of *Nothobranchius* mortality (Haas 1976a). It was logistically unfeasible to include site-specific observation of bird presence or reliably quantify their visitation rate to our fieldsites because bird visitation of sites is typically unpredictable. However, we have indirect evidence of their widespread presence. A parasitological examination of 14 *Nothobranchius* populations across our study area revealed a high prevalence (93 %) of several species of digenean larvae (platyhelminths) (V. Michálková, unpublished data). Fish are intermediate hosts of digenean parasites, with fish-eating birds the definitive hosts, discharging parasite's eggs into the pools in their faeces (Poulin 2007). Hence, circumstantial evidence suggests fish-eating birds visit *Nothobranchius* pools regularly.

We recorded several cases of piscivorous birds hunting in pools we collected from, including kingfishers, herons, storks and hammerheads. Some fish were injured with clear signs of bird attacks on their bodies (Fig. S2). Thus we consider bird predation as an important source of fish mortality. In an experimental setting, a higher rate of bird predation was recorded on fish with specific colour patterns (Maan et al. 2008), including on brightly coloured males of *Nothobranchius guentheri* (Pfeffer) (Haas 1976a). At the same time, we acknowledge that the pools with abundant vegetation had markedly clearer water, making *Nothobranchius* males more visible and hence more likely to be apparent to visual predators.

Direct male–male competition often has serious consequences for rivals, irrespective of dominance (Le Boeuf 1974; Hutchings and Myers 1987). This is also the case in *Nothobranchius*, where males aggressively compete for access to females (Haas 1976b), leading to male mortalities in captivity (e.g. Genade 2005). Mortality related to male–male contests can be intensified when subordinate males have fewer opportunities to escape from dominant males. Accordingly, subordinate males in structurally simple habitats, with little or no vegetation, may have been more prone to fatal injuries from dominant individuals than those in more structurally complex habitats where refuges were available. Hence, vegetation cover may reduce the rate of male mortality resulting from male–male competition. While these possibilities are theoretically plausible and could be tested experimentally, ethical concerns effectively preclude such experiments.

Finally, males are often more vulnerable than females to environmental disturbances and food shortage, resulting in bouts of severe male mortality (Clutton-Brock et al. 1991; Clutton-Brock and Coulson 2002; Arendt et al. 2014), but our study was not designed to examine this possibility.

Consequences for sexual selection and demography

Identifying the source of male mortality is important. For example, any potential effect of elevated male mortality on sexual selection will depend on the cause of male mortality. If predation is the chief cause of sex bias, the prediction is that the most colourful and active males are likely to be the targets of selective predation. Consequently, predation will tend to reduce variation in fitness and reduce Fisherian sexual selection. For example, strongly male-biased predation on agile frogs (*Rana dalmatina*) reduced incidences of multiple mating and alternative male mating behaviour (Lodé et al. 2004). In contrast, if male mortality stems from competition among males for mating opportunities, subordinate males are predicted to suffer the greatest mortality, thereby elevating the strength of sexual selection, as reported in elephant seals (*Mirounga augustirostris*) (Hoelzel et al. 1999). A strong female-bias or its seasonal increase may have additional consequences for sexual selection and reproductive rate. It can lead to a reversal of sex roles whereby females compete fiercely for access to males (Forsgren et al. 2004; Liao et al. 2014), modulating the reproductive success of particular phenotypes (Jirotkul 1999; Reichard et al. 2004a) or even the entire population (Reichard et al. 2004b).

Male selective mortality has a relatively smaller effect on population viability when the mating system is polygynandrous or promiscuous (as in *Nothobranchius*) (Boukal et al. 2008). Nevertheless, we recorded extremely female-biased sex ratios in some populations. For example, considering populations with sample sizes larger than 30 adult fish (where chance effects of sampling should be minimized), we observed 7 cases (10 % of populations) with a female bias of 1:10 (sex ratio 0.10) or higher in *N. furzeri*, including sex ratios of 0.02 (1 male, 43 females), 0.03 (1 male, 35 females) or 0.04 (2 males, 43 males), and comparable results for *N. orthonotus* and *N. rachovii*. Such sex ratios may have direct costs in reproduction as a result of the inability of some females to find a mate. We repeatedly observed females with large swollen abdomens in the most female-biased populations, while no such phenotypes were encountered in other populations. Therefore, a low abundance of males may have profound population consequences by decreasing female oviposition rate.

General implications

Recent research on the mechanisms and consequences of adult sex ratio biases in vertebrates (Arendt et al. 2014; Székely et al. 2014a, b, present study) converges on the following conclusions. First, the biases arise from sex-specific mortality in adults. Second, the factors underpinning sex-specific mortality are often complex. For example, our study revealed that a superficially straightforward effect of sex-biased predation is modulated by multifarious interactions with environmental settings. Third, there is a high level of stochasticity and large datasets are needed to provide robust insight. Fourth, even closely related sympatric species may vary in adult sex bias. Fifth, sex ratio bias is dynamic; there are often bouts of increased sex-specific mortality at particular periods rather than successive stable disappearance of one sex from the adult population due to its consistently higher mortality. The seasonal dynamic in adult sex ratio then becomes very complex when age cohorts overlap (Arendt et al. 2014). Finally, the source of sex specific mortality is important because it may target specific phenotypes (e.g. large or small, bold or shy, territorial or subordinate males), with important evolutionary consequences. Current understanding of phenotype-specific mortality within the more susceptible sex is limited.

Future studies should examine phenotype-specific mortality to further illuminate the evolutionary significance of adult sex ratio biases.

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