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Sex ratios deviate across killifish species without clear links to life history

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Abstract
Sex ratios can differ from an expected equal proportion of males and females, carrying substantial implications for our understanding of how mating systems evolve. Typically, macro-evolutionary studies have been conducted without assessing how deviations from an equal sex ratio could be explained by sex-biased mortality or dispersal. Our understanding of sex ratio evolution independent of these confounds, in addition to any putative links between skewed sex ratios and other factors (e.g. life history), therefore remains largely unexplored. Here, we conducted an exploratory study investigating differences in sex ratios across closely related species while controlling for extrinsic mortality. We also tested two factors, non-overlapping/overlapping generations and the social environment, which have both been hypothesised to affect sex ratios. Specifically, we raised 15 species of killifish, which have either overlapping or discrete generations, under both solitary and social treatments. We found substantial divergences in sex ratios across closely related species, which exhibited both male and female biases. In conjunction with a low phylogenetic signal, our results suggest that sex ratios can evolve rapidly in this group. However, we found no evidence that overlapping generations or the social environment affected sex biases, suggesting that other factors drive the rapid evolution of sex ratios in killifishes.

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Introduction

Classic evolutionary theory predicts an equal sex ratio, with negative frequency dependent selection acting on the excess production of any one sex (Düsing 1884; Fisher 1930). However, across gonochoric taxa, sex ratios can be highly variable, with the cause of this variation suspected to differ widely (West and Sheldon 2002; Donald 2007; Liker et al. 2013; Székely et al. 2014a). For instance, sex can be determined genetically and/or even plastically, in response to environmental cues (Trivers 1985; McNamara et al. 2000; Le Galliard et al. 2005; Kokko and Jennions 2008; Székely et al. 2014a). Importantly, any variation in sex ratios across taxa carries substantial evolutionary relevance, influencing sexual selection (Janicke and Morrow 2018), and by playing a ubiquitous role in shaping population demographics and mating systems.

Empirical studies on the evolution of sex ratios, particularly at macro-evolutionary scales, have typically been biased towards the adult (ASR) or operational (i.e. ratio of sexual receptive adults) sex ratio (e.g. Pipoly et al. 2015). While such studies are informative for the evolution of mating systems, our understanding of the key mechanisms driving evolutionary distortions in the initial sex ratio remains limited. Specifically, ASRs are likely influenced by secondary factors, such as sex-specific survival (Arendt et al. 2014; Székely et al. 2014b), time to sexual maturity, and sex specific dispersal patterns (Bessa-Gomes et al. 2004; Veran and Beissinger 2009; Reichard et al. 2014). Adult sex ratios are therefore not necessarily a reliable measure of the initial sex ratio when estimated in natural systems, even if the ASR evolved as a function of the initial sex ratio, which in turn, is determined by zygote formation or sex-allocation (see Clutton-Brock and Parker 1992).

Indeed, secondary factors such as sex specific mortality can exert a considerable influence on sex ratios (Tabadkani et al. 2013; Ancona et al. 2017). For instance, Tabadkani et al. (2013) found a striking change in the sex ratio of the predatory gall midge (*Aphidoletes aphidimyza*) over the first few days after a simultaneous hatching event. At hatching, the sex ratio in this species is slightly male biased (52.41% male), but within 4 days the population shifts to an extreme female bias (97% female). This striking difference indicates that sex ratios can change rapidly within a cohort, and that sex ratios estimated under natural conditions will be predisposed to reflect sex-specific mortality. Hence, while we have considerable knowledge on which factors influence ASRs (often intentionally), fundamental aspects of sex ratio evolution remain largely unexplored. This lack of knowledge includes how commonly sex ratios deviate from equality, how fast deviations can evolve, and any links between life history and the evolution of skewed sex ratios.

In nature, sex ratios may deviate among lineages due to differences in sex determining mechanisms, including plastic sex determination in response to variability in environmental conditions. For example, in many taxa, sex determination can be plastically influenced by environmental factors, such as temperature during the embryonic stages. Indeed, phylogenetic analyses suggest frequent evolutionary transitions between genetic and temperature-dependent sex determination (Bull and Charnov 1989; Olsson and Shine 2001; Mank and Avise 2009; Sarre et al. 2011, although see Ospina-Álvarez and Piferrer 2008). While the mechanisms that modulate sex determination may be adaptive, even minor changes to the environmental conditions under which environmentally sensitive organisms have been selected can easily distort sex ratios, even compromising the survival of these populations (Jaenike 2001; Warner and Shine 2008; Holleley et al. 2015). Similarly, social factors may directly influence sex determination (Guerrero-Estévez and Moreno-Mendoza 2010). For instance, in the coral-dwelling fish, *Gobiodon*

erythrospilus, the presence of conspecifics can plastically induce the direction of sex determination, with juveniles maturing into the opposite sex of the first individual they encounter (Hobbs et al. 2004). Importantly, greater susceptibility to environmental influences can impose selective pressures by distorting initial sex ratios away from equality, therby potentially affecting the evolution of underlying genetic sex determining mechanisms (Mank et al. 2006).

Skewed sex ratios are directly linked to both the demography of a population, and the sex determination system. Sex determination is also likely influenced by the life-history characteristics of an organism, such as sex-specific survival, fecundity, or the presence or absence of overlapping generations (Charnov 1982; Hamel et al. 2016). However, life-history traits are often not considered in theoretical models of sex determination or allocation, and hence, sex ratio evolution. For example, theoretical models have typically focused on systems with discrete generations, despite overlapping generations being more common across animals (Shaw and Mohler 1953; Bodmer and Edwards 1960; Kolman 1960; Crow and Kimura 1970; Charnov 1982; Komdeur and Pen 2002). Nonetheless, there is theoretical and empirical evidence, albeit limited, that does suggest that generation overlap may influence sex allocation, and sex ratios (Werren and Charnov 1978; Kahn et al. 2013). For instance, in species with overlapping generations, temporal changes in the fitness expectations of the sexes, such as sex-specific differences in seasonal survival (commonly observed in nature; Smallwood and Smallwood 1998; Bowden et al. 2000), can allow selection for the temporal overproduction of one sex (Werren and Charnov 1978; Kahn et al. 2013). In such an event, sex-biased mortality in one generation can lead to subsequent biases in the opposing direction. This has been observed, for example, in the mosquito fish *Gambusia holbrooki*, where seasonal biases for females can occur when males are unlikely to survive the winter (Kahn et al. 2013). Hence, the presence of overlapping generations does not favour selection for biased sex ratios per se, but may instead create scope for such divergences in sex ratios to occur (e.g. in response to differential sex-specific survival).

To assess if deviations from an equal sex ratio can evolve rapidly, we tested for divergences in sex ratios across closely related species, under conditions that control for sex-specific mortality. Further, we tested whether the presence of overlapping or non-overlapping generations was linked to any deviations from an equal initial sex ratio. We did this because deviations from an equal sex ratio could be adaptive, for example, in situations of regular sex-biased mortality (e.g. seasonal mortality), which is well documented in many fishes (Balshine-Earn 1995; Godin 1995; Quinn and Buck 2001). We employed killifishes (15 species) as our model system, as they display extreme divergences in life-history traits between annual and non-annual species, including reproductive rate (8 times higher in annual species, Eckerström-Liedholm et al. 2017), growth rate (1.3 times higher in annual species, Sowersby et al. 2019), life-span (shorter in annual species, Genade et al. 2005; Blažek et al. 2013; Berois et al. 2015), and importantly generation overlap. These differences in life-history traits have occurred largely independently from behavioural changes (Eckerström-Liedholm et al. 2019), but instead as a consequence of several independent evolutionary transitions to inhabit seasonally ephemeral water bodies (Furness et al. 2015). Here, we reared fry in either solitary or social tanks. As social factors have previously been found to affect sex ratios in fish (Hobbs et al. 2004), we also controlled for this social treatment in our analyses (i.e. solitary or exposed to conspecifics). Although, we did not have any a priori expectations in respect to how or in which direction the social treatment would affect the sex ratios of the different species.

We assessed the sex ratio of each species only when individuals could be accurately sexed by visual inspection (i.e. at sexual maturity). We did this because sex determination

mechanisms in many teleosts (including cyprinodont fishes) either remain unknown, or are highly evolutionarily volatile, with multiple transitions existing between genetic and plastic mechanisms even amongst closely related species (Yamamoto 1969; Devlin and Nagahama 2002; Mank et al. 2006; Baroiller et al. 2009; Guerrero-Estévez and Moreno-Mendoza 2010). For this reason, sex determination in fishes can often be delayed until several days or even weeks post hatching, delaying considerably any possible estimates of sex ratios in comparison to other vertebrate taxa (Mank and Avise 2009).Theoretically, this potential delay in sex determination might coevolve with an evolutionarily labile sex determination mechanism in fishes (approximately 10% of fish species have been found to have hetero-morphic sex chromosomes, sensu Devlin and Nagahama 2002), which could increase the likelihood of sex ratios evolving in concordance with life history variation and environ-mental cues. This is in comparison to taxa with more deterministic sex mechanisms, such as non-recombining sex-chromosomes (Bachtrog et al. 2014).

Materials and methods

Experimental set-up

We raised killifish from 15 species (Suborder Aplocheiloidei), which differ in key life-his-tory traits (n=6 annual species, n=9 non-annual species, as per Furness et al. 2015, see Supplementary Fig. 1, mean 32 individuals per species across treatments, range 15–43), to investigate if any deviations from equal sex ratios are associated with differences in tempo-rarily overlapping generations (i.e. life-history strategy). In addition, we explored whether the social environment, in particular the presence of conspecifics can influence sex ratios among species. The social treatment was largely included due to logistic constraints, as the number of individuals needed to determine deviations from an equal sex ratio is high, and it was not feasible to rear an appropriate number of individuals for all species under solitary conditions. However, as some fishes are known to exhibit plasticity in sex ratios in response to social conditions (e.g. Hobbs et al. 2004), we accounted for this treatment in our analyses.

After hatching, fish were kept separately in smaller plastic containers (0.75 L) for 1 week, to ensure that all individuals were feeding independently. Fish were then moved to their rearing conditions, i.e. raised in either a social isolation treatment (n=172 individu-als) or in a social group treatment (n=40 groups, 293 total individuals, density of 7–8 con-specifics per social treatment). In all other respects, apart from stocking density of social treatments, housing conditions were standardised. All tanks (size 13 L), were kept at an average temperature of 24.3 °C (range 22.6–25.1), under a 12-h day-night cycle, and all fish were fed a mixture of newly hatched *Artemia* and commercial frozen Chironomids, ad libitum three times daily (once per day during weekends). Water chemistry was meas-ured periodically and kept stable via regular water changes. Aquaria contained a floating "mop" made of dark coloured acrylic yarn and a small terracotta pot to provide shelter. This experiment ran between February 2016 and September 2017, at the Department of Zoology, Stockholm University.

All fish were hatched in the laboratory from eggs from our own laboratory popula-tion stock or from dedicated killifish breeders. Elsewhere, sex-biased mortality has been observed under captive conditions in birds and mammals (e.g. Bradbury and Blakey

1998; Baxter et al. 2012). However, here survival was very high, near 100% in most species (except for *F. filamentosus,* which had minor levels of juvenile mortality), including in those species that exhibited strong sex biases. Unfortunately, we could not confidently estimate egg mortality under our laboratory setup, although in many fish species sex is not determined until after hatching (Mank and Avise 2009). While we also cannot totally exclude the possibility that conditions during egg transportation resulted in sex biases, this is unlikely, as we did observe sex biases in species produced solely from our laboratory stock. Increased temperature has previously been found to affect sex ratios in fish (Abozaid et al. 2011), although this is viewed as an exception, which generally generates a surplus of males (Ospina-Álvarez and Piferrer 2008). However, we did use many species that are naturally distributed across different latitudes (i.e. with different mean ambient temperatures) and housed them all under the same temperature regime. We hence investigated if a difference between source and laboratory temperature could have had an effect on the sex ratios that we observed. Specifically, if sex ratios constitute a species-specific plastic change to thermal conditions, we would expect the sex ratio to be optimized (at equal sex ratios) in their respective natural thermal conditions. Further, if this was the case, skewed sex ratios that differ from unity when reared under standardized conditions, should correlate to the species-specific natural thermal conditions. To test for this, we correlated each species' original collection location as a proxy for natural temperature (latitude, from Killi-Data http://www.killi-data.org, Supplementary Table 1) with the observed sex ratio in the laboratory. We did not find a significant correlation between collection latitude and biases in sex ratios observed under laboratory conditions (Pearson's correlation, $r_{13} = 0.32$, $p = 0.23$). Finally, we also assessed whether the time a species had been kept in captivity (as a proxy for potential inbreeding and domestication) was associated with biases in the sex ratio. We performed this analysis specifically because there is evidence suggesting that zebrafish strains, *Danio rerio*, have evolved various sex determining mechanisms in captivity, and that the sex genotype does not always match the sex phenotype (Wilson et al. 2014). We found no correlation between the collection date (i.e. time spent in captivity) and any bias in the sex ratio for the eight species for which there was available data (see Supplementary Table 4; Supplementary Fig. 2), suggesting that time in captivity has not influenced our results.

Table 1 Model output testing the effects of life-history strategy (Annual or Non-Annual) and social treatment (Solitary or Group) on the sex ratio of 15 killifish species

Parameter	Estimate	Lower CI	Upper CI	P_{MCMC}
Fixed effects				
Intercept	0.46	−0.53	1.59	0.36
Social environment	0.38	−0.47	1.13	0.31
Life-history strategy (non-annual)	−0.14	−1.24	1.12	0.79
Social environment: life history strategy (non-annual)	−0.97	−1.93	0.02	0.06
Random effects				
Species	0.31	1.45×10^{-05}	1.21	
Phylogeny	0.003	5.99×10^{-06}	1.41	
Residual variance	1	1	1	

Random effects are estimates of variances

The fish used in this study were collected as eggs, which were produced in stock tanks containing a large number of breeding individuals. Therefore, the eggs used in this experiment came from multiple mothers and fathers, reducing any effects driven by potential differences in specific paternal phenotypes. Specifically, as individuals used in this study came from eggs collected from multiple pairs per species (from a minimum range of 2–9 pairs in our laboratory, for 11 of the species, Supplementary Table 3). Eggs were subsequently pooled, to get a representative value for species-specific sex ratios. However, the actual number of parents was higher, with additional eggs (for 12 species, mean number of additional eggs = 47 per species) supplied by hobbyists. In addition, killifishes lay eggs sequentially rather than in clutches, meaning that females can easily have offspring with multiple males. Eggs collected from both our laboratory and hobbyists were used across both experimental treatments. As per above, immediately after hatching, all killifish were kept solitary in small containers (0.75 L, 9×9×9 cm), for approximately 7–10 days, after which they were transferred to the larger experimental aquaria. All group members were hatched within 5 days of each other and added to the treatment tank as juveniles.

Experimental conditions were maintained until an individual or all group members displayed the species-specific phenotypic traits that distinguish the sexes. As sex is not determined in many fishes until well after hatching, we assessed sex ratios when fish became sexually mature. Killifishes are typically sexually dimorphic, and while the degree to which males and females differ is species specific, all individuals could be scored as either male or female from visual inspection. To confirm the accuracy of our visual inspections, as a method for assigning sexual phenotypes, 335 individuals distributed over all species were euthanized (lethal dose of benzocaine solution) and dissected to directly observe the gonad tissue. In all cases our visual assessment of sex based on sexual dimorphism was accurate.

Statistical analysis

Modelling

The proportion of males in each species was analysed using a linear mixed model, where sex was coded as a binary variable (females = 0, males = 1). A Bayesian approach using a Gibbs sampler implemented in the R package MCMCglmm (Hadfield 2010) was used for all analyses. As we were interested in the effect of species and life-history strategy (annual or non-annual), in addition to the potential effect of social treatment (solitary or social), we modelled sex as being dependent on the fixed effects: life history, social treatment and their interaction. Independent species effects and correlated phylogenetic species effects based on a dated phylogeny (Furness et al. 2015; Fig. 1; Supplementary Fig. 1), were added as random effects to the analysis. The putative effect of tank identity was tested as a random effect (by adding an incidence matrix of tank identities within the group reared fish as a random effect), but it was excluded as it increased DIC values (delta DIC ~ 1). The residual variance was fixed to 1. Accurate estimations of sex ratios are likely to frequently suffer from low statistical power (Booksmythe et al. 2013). It was hence important to assess that species identity explained a significant part of the variation in the sex ratio. To assess the validity of a species effect, we compared the DIC values of the Bayesian models that excluded the species effects. The Bayesian model was run with 4×10^6 iterations, where 8×10^5 iterations were discarded as burn-in. A thinning interval of 800 was used yielding a total posterior sample of $3\times4000 = 12,000$ iterations. We used rather flat priors for the fixed effects and locally uninformative priors for the random effects, to represent little
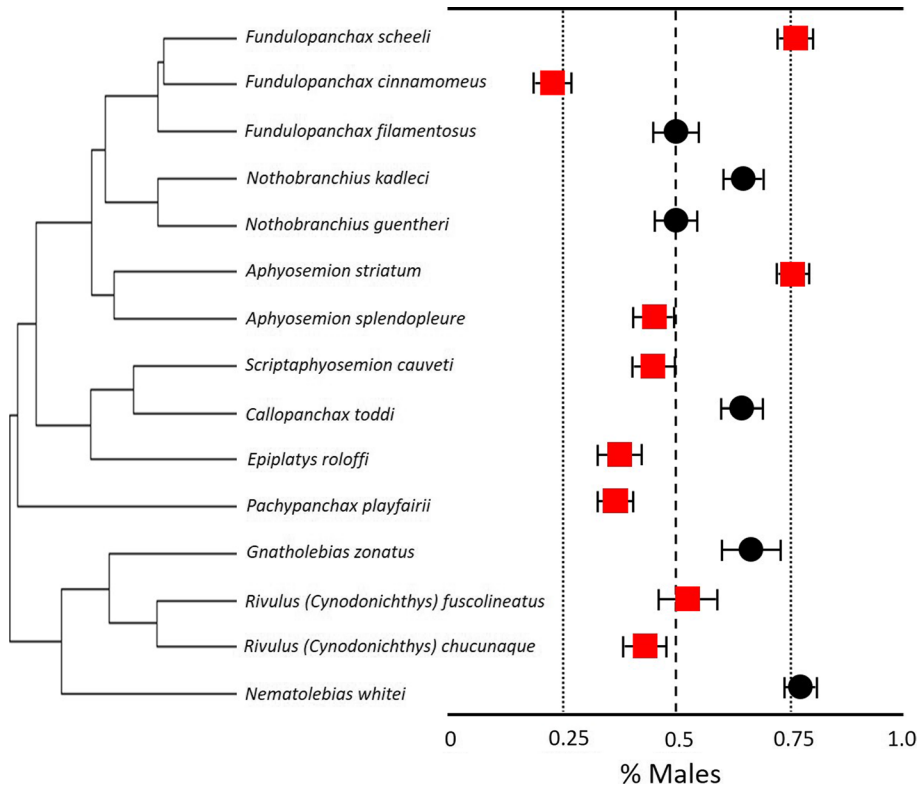
Fig. 1 The time calibrated phylogeny (Furness et al. 2015) and species used in this study. Sex bias per species are shown on the right (calculated from raw values, higher values indicate a male biased sex ratio). Red squares indicate non-annual and black circles indicate annual species. Point estimates are the highest posterior. Whiskers indicate standard error

prior knowledge. All estimates of the autocorrelations between successive posterior samples were within the interval [−0.1, 0.1].

It should be noted that DIC values are contested as a measure of model fit for complex Bayesian models (Spiegelhalter et al. 2014; Hooten and Hobbs 2015). It is hence not trivial to test the inclusion of a random effect in a generalized linear mixed model. Sex ratios are prone to sampling errors, and as our intention was to assess species level effects, we needed to be confident that any species level effects were not driven by sampling variance within species. Hence, to further validate species effects, we fit a generalized linear model using maximum likelihood (Laplace Approximation) as implemented in the lme4 package in R. Here, we used a dummy variable, consisting of a resampling of the species identities (i.e. breaking the bond between an individual's sex and species), in addition to the real species identities. We fit one model containing both the true species identities and the dummy variable as random effects, and a second model containing only the dummy variable. The resampling of the dummy variable was performed 1000 times to get a valid approximation of the significance of the species effect. Under the null hypothesis, approximately zero variance should load on these random effects, and the effect of species should be non-significant. The two models were then tested against each other, and a significant

effect was interpreted as the species effect having improved the fit of the model. Finally, we used another simulation approach to assess if variance across families, within species, could distort the patterns that we observed. We did this because we had a rather low number of families per species, and family effects (either plastic or genetic) could induce family level sampling error. This sampling approach was based on the sex ratios and number of families of two particular species (*Pachypanchax playfairii* and *Aphyosemion striatum*) for which all individuals were produced from eggs from our laboratory population under standardized conditions. Here, we allowed each individual to be randomly sampled from a given number of families (*A. striatum* n = 9 and *P. playfairii* n = 7 families), where family effects (the family intra-class correlation, "ICC", similar to a heritability) varied between 0.01 and 0.95. We used a set total variation, estimated from the latent scale residual variation, extracted from the model sex ~ species (V ~ 1.2). Values were simulated on a logit scale, where family effects were sampled from a normal distribution with mean = 0 and variation family ICC × V. The individual level simulated values were sampled from a normal distribution with mean = a vector of family means and variance = 1-ICC × V. The simulated values were transformed into probabilities using the inverse logit, that subsequently were used to sample a binomial distribution for our response, sex. On 1000 simulated datasets, per value of family ICC, we tested type I error rate (occurrence of false significance), and how often the simulated datasets had effects larger than the effect we observed in our actual dataset.

Testing for links with generation overlap

While we had no a priori assumption for sex ratios deviating from 0.5 to be either female or male biased, we conducted an exploratory test of whether an annual life history, with non-overlapping generations, would correspond to a more equal sex ratio. Hence, we needed to estimate the effects on an absolute level. Utilizing the fact that the latent scale of the generalized linear models is assumed to be normal, we applied a method where Gaussian parameter estimates give expected values on a 'folded-over' distribution (Hereford et al. 2004; Morrissey and Hadfield 2012) given by:

$$E(y) = \sigma \times \sqrt{(2/\pi)} \times \exp\left(\frac{-|\mu|^2}{2\sigma^2}\right) + |\mu| \times (1 - 2\Phi \times (-|\mu|/\sigma))$$

where $E(y)$ is the expectation on the folded-over distribution, σ the estimated standard deviation, μ the mean and Φ denotes the cumulative distribution function of the standard normal distribution. Note that this approximation allowed us to test simultaneously for effects on the mean and variance in sex ratios (as departures from an equal sex ratio, in any direction, will have the same effect on absolute values). The required variances were obtained by a new model fitting only an intercept, and separate species variances for each level of the factors "Life history" and "Social treatment". The residual variance, fixed to 1, plus the link specific variance for the logit fit of binomial data, pi[π] $^{2/3}$, were included in the calculations (Nakagawa and Schielzeth 2010).

Results

Sex ratios differed across species, with the percentage of males varying between 23% in *Fundulopanchax cinnamomeus* (se ± 0.078) to 76% (se ± 0.073) in the closely related *F. scheeli* (averaged across both treatments; Fig. 1). Species explained a strongly significant proportion of the variance in sex ratio, and was highly significant when tested for inclusion in the maximum likelihood model against a resampled dummy variable, where the *maximal p* value for the 1000 iterations of resampling was 0.002, corresponding to a minimal χ^2 of 9.4. The median *p* value and χ^2 across the simulations was 0.0007 and 11.5, respectively. When assessing if sampling variance on families could have affected our results, we found that there could be moderate family effects, which would strongly increase the type I error rate in finding false positive species effects (at a family ICC of 0.4, the type I error rate (using alpha < 0.05) was around 10%). However, we do not believe that family effects were driving the species level effects that we report, as our simulated false positives only reached the magnitude of the species level effects in 1.5% of cases, and only at family ICC's of an unrealistic 95%. The significant species effects were validated by credibility intervals (Fig. 1), and based on the fact that species also improved the fit in the Bayesian model (delta DIC = 2.5: note that delta DIC needs to be interpreted with caution Spiegelhalter et al. 2014; Hooten and Hobbs 2015). The estimates of variance components for both species and phylogenetic effects in the Bayesian model were small (species 0.065, 95% CI 0.0003; 0.23, phylogenetic heritability = 0.059; 95% CI 0.0001; 0.27). Large divergences across closely related species in combination with the relatively low number of species included (n = 15), made it difficult to disentangle the relative effects of species and phylogeny. A model containing only species yielded a similar coefficient of variation, but with narrower credibility intervals (0.08, 0.017, 0.24). Note that this is in accord with the low phylogenetic signal observed in the model accounting for phylogeny. Our main model showed no significant effects of life history or social treatment on sex ratio, either in the model output, or when testing absolute sex-bias with a folded normal distribution (Table 1; Fig. 2).

Fig. 2 Sex ratio changes across social treatments. The red squares indicate non-annual and black circles indicate annual species. Point estimates are the medians of the posterior distribution, and the corresponding error bars the 95% credibility intervals
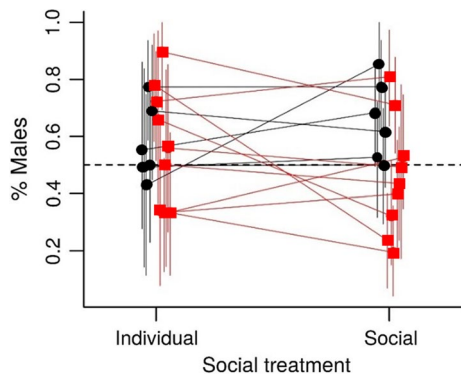
Discussion

We conducted an exploratory, comparative analysis, on the sex ratio of 15 species of annual and non-annual killifish. We documented large divergences across species and found that skews in sex ratios were either male or female biased. In addition, we found a low phylogenetic signal, with different sex ratios occurring between closely related species, suggesting that deviations from expected sex ratios can evolve rapidly. However, we did not find any indication that the presence or absence of overlapping generations (i.e. non-annual or annual life-history strategy) or social factors (i.e. solitary or social treatment) affected observed sex-biases, implying that these factors may have a minor importance in shaping sex ratios in this system.

Deviations from equality in sex ratios at birth have been observed in a range of other taxa (marsupials: Cockburn 1989; Davison and Ward 1998; birds: Nager et al. 1996; Whittingham et al. 2005; Alonso-Alvarez 2006). Furthermore, deviations from equal sex ratios are potentially adaptive, as shown in parasitic wasps, where females gain a greater benefit from a larger adult body size, which is highly dependent on initial host size. In these wasps, it is therefore favourable for mothers to ensure that daughters are placed in relatively larger hosts, compared to sons (Cook et al. 1994), with differences in host size leading to an adaptive bias in sex ratios. Alternatively, shifts in sex ratios may be caused by environmental interference with sex determining systems (Römer and Beisenherz 1996). However, to date, a majority of studies have been conducted under natural conditions, where secondary factors such as sex specific survival and sampling errors due to sex specific behaviour may bias observations. The evolution of initial sex ratios (i.e. independent of sex specific mortality) compared to ASRs is therefore rather unexplored, especially on a comparative scale, which we show here can shift from an expected 1:1 ratio.

Generation overlap is one life-history characteristic that may influence the evolution of sex ratios and theoretically allow shifts in the sex ratio away from 1:1 (Charnov 1975; Werren and Charnov 1978). Such shifts could occur and be adaptive, for example, in combination with differential seasonal survival of the sexes (Werren and Charnov 1978; Kahn et al. 2013). However, despite the theoretical expectations that skews in sex ratios could evolve in systems with overlapping generations, we did not find any support for this prediction. Nonetheless, we found evidence against the established prediction that selection should typically yield an even initial sex ratio (Fisher 1930; Shaw and Mohler 1953; Bodmer and Edwards 1960; Crow and Kimura 1970). Indeed, we found remarkable differences in sex ratios, even between closely related sister taxa. For example, we observed a proportion of 0.23 males in *F. cinnamomeus*, compared to a highly male biased 0.76 in the closely related *F. scheeli* (averaged across both social treatments). Such volatility in sex ratios, between closely related species, suggests that the evolution of sex ratios can occur rapidly. While we could not confidently estimate egg mortality, in many fish species sex determination is delayed until close or after maturation, allowing external factors to play a proximate role long after birth/hatching (Devlin and Nagahama 2002; Mank et al. 2006). Developmentally, testes and ovaries in teleosts all derive from the same precursor tissue and can rather flexibly differentiate at various life stages within an individual (Devlin and Nagahama 2002). In our study, all species were kept under identical conditions, implying that if environmental effects did influence sex-determination, such plastic effects must have been species-specific. We have no clear a posteriori explanation for the observed sex biases in several species of killifish, and what drives these differences remains enigmatic. Nonetheless, our study is one of very few (if not the first) to demonstrate that sex ratios deviate readily at a macro-evolutionary level under controlled conditions (i.e. by

controlling for mortality in the laboratory). We acknowledge that external factors such as temperature have the potential to skew sex ratios. However, as our study investigated evolved differences in a common garden setting, we could not address how any putative plastic responses would translate to distorted sex ratios in the wild. Nevertheless, our study demonstrates that deviations from expected sex ratios can occur readily, but due to the general lack of knowledge regarding the mechanisms behind sex determination, additional studies are needed to elucidate the factors that give rise to such striking differences in sex ratio. We also acknowledge that laboratory conditions clearly differ from natural conditions, and that factors in the natural environment could influence sex determination, resulting in more equal sex ratios in nature. While we attempted to test for any potential effects caused by the lab temperature differing from source temperatures, we cannot make any inference regarding other environmental factors (such as resource levels, predators, or other physicochemical factors). A promising avenue for future research would be to use reciprocal transplant experiments to identify environmental conditions that are putatively important for sex-determination, and to test these environmental conditions in depth using common garden experiments with multiple treatments.

Biases in sex ratios are likely to be tightly linked to divergences in sex determination systems. A larger scope for biases in the sex ratio may therefore be more likely among organisms with more plastic sex determination mechanisms, or in clades with frequent evolutionary changes between sex-determination mechanisms. Under this scenario, we could reasonably expect greater flexibility in the selection of sex ratios according to the life history of the organism. The Actinopterygii, including killifishes, contains species with a diverse array of sex determination mechanisms, likely more varied than any other vertebrate group (Mank et al. 2006). Among the killifishes included in our study, at least two species are known to have sex chromosomes, specifically the annual species *Nothobranchius guentheri* (*N. guentheri* has a multiple sex chromosome system; Ewulonu et al. 1985) and the non-annual *Aphyosemion splendopleure* (Völker et al. 2008). Interestingly, *N. guentheri* was observed to have 0.5 sex ratio, suggesting that genetic sex determination, via the sex chromosomes, typically results in a balanced sex ratio. In our study, regardless of the mechanism responsible for skewed sex ratios, differences across even closely related species are observed, suggesting sex ratios can evolve at a rather rapid rate. More generally, our knowledge about the sex determining mechanisms in many fish species, including most killifishes, remains limited. In other taxa, chromosomal sex determination is not necessarily regarded as a major constraint to minor sex ratio adjustment (West and Sheldon 2002). This is particularly apparent in birds, where females are the heterogametic sex (Pike and Petrie 2003; Alonso-Alvarez 2006; Rutkowska and Badyaev 2008), and biased segregation of chromosomes during female meiosis has been proposed as a main mechanism for sex ratio adjustment (Rutkowska and Badyaev 2008). As such, additional research into the mechanisms that determine sex in fishes would allow for inferences on how different modes of sex determination contribute to biases in sex ratios, observed across species.

Conclusion

In conclusion, we observed significant deviations away from a predicted 1:1 sex ratio in several killifish species. However, these observed skews in sex ratios did not appear to be influenced by either difference in generation overlap or by social factors. Importantly, our study represents one of the first investigations into the evolution of sex ratios, assessed under conditions controlled for sex-specific mortality, at a macro-evolutionary level. We

show that under controlled conditions, sex ratios can differ remarkably between closely related species, suggesting that they are not constrained by phylogeny and can change rapidly, to the extent that both adult and operational sex ratios may be affected. Importantly, we advocate for further research into the evolution of sex ratios, especially on the factors which may facilitate selection away from an equal ratio of males and females in taxa where sex determination is not under direct genetic control. Further research is especially important in taxa that may be vulnerable to rapid, human induced environmental changes, which can influence the balance of sex ratios and shift fundamental population demographics.

Compliance with ethical standards

Ethical approval This research was conducted with the approval of the ethics committee, Stockholm University (ethics permit number N132/15), and complied with Swedish law for animal experiments (L150).

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