ORGANISMAL BIOLOGY

Complex effects of sex reversal on reproductive success in wild frogs

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Sex reversal, when the environment overrides genotypic sex determination, may hasten or delay climate-driven extinction in ectothermic animals, depending on sex-reversed individuals' reproductive ability and offspring viability. Empirically, next to nothing is known about these traits in natural populations. Here, we present a comprehensive dataset of wild frogs to compare sex-reversed and sex-concordant males for fertility, attractiveness to females, intrasexual competitiveness, and offspring survival in various environments. We demonstrate uncompromised potential of sex-reversed males to mate and produce viable offspring, which are all genotypic females but have increased sex-reversal propensity. However, sex-reversed males realize reduced paternity in natural populations, possibly due to cryptic female choice. Furthermore, offspring of sex-reversed sires exhibit increased vulnerability to viral infection after heat stress. Our theoretical model predicts that these handicaps of sex-reversed males compromise their ability to compensate by producing female offspring for climate-driven male bias in phenotypic sex ratios, which reduces population viability. Therefore, sex reversal exacerbates the biodiversity consequences of environmental change.

INTRODUCTION

After finding half a century ago that sex is determined by environmental factors in some reptiles and fish (*1*, *2*), the advancement of genomic techniques over the past two decades allowed us to realize that the natural environment can influence sex also in species that have genotypic sex determination (*3*, *4*). This environmental override results in a mismatch between phenotypic and genotypic sex, a peculiar phenomenon termed sex reversal. A vast body of laboratory experiments shows that many species are susceptible to sex reversal induced by environmental temperatures and chemicals (*5*–*7*). Sex-reversed individuals may be widespread in nature because they have been found whenever they were looked for in published studies of wild populations of amphibians, reptiles, and fish (*5*, *8*, *9*). Theoretical models predict that thermal sex reversal has wide-ranging consequences for population dynamics, microevolution, and phylogeography, particularly under persistent climatic warming (*4*, *10*–*14*). These predicted consequences critically depend on the mating success and fertility of sex-reversed individuals and the viability of their offspring, ranging from scenarios where climate-driven sex reversal exterminates the population to scenarios where a higher participation of sex-reversed individuals in reproduction mitigates extinction risk (*10*–*13*). For example, because the offspring of sex-reversed individuals have biased genotypic sex ratios, they can mitigate the skewing of phenotypic sex ratio in the population and therefore postpone the crashing of effective population size; however, this mitigating effect is lost if sex-reversed individuals are not able to contribute to the next generations (*10*–*13*). Therefore, quantifying the breeding performance of sex-reversed individuals is crucial for understanding and forecasting the effects of anthropogenic environmental change across the diversity of taxa that exhibit environmentally sensitive sex determination. Because these taxa include high percentages of threatened species, e.g., 41% in amphibians (*15*), this issue is of particular importance for biodiversity conservation.

Empirically, however, only a tiny fraction of extant species has been studied for the evolutionary ecology of sex reversal (*9*, *16*), so data on the reproductive performance of sex-reversed individuals in natural systems are extremely scant. Reduced reproductive success may be expected for sex-reversed individuals because of sex-antagonistic alleles accumulating on sex chromosomes, although the latter idea is challenged by empirical data from species with sex-chromosome polymorphisms (*17*, *18*). Aquaculture data suggest that sex reversal induced by hormonal treatment reduces multiple aspects of breeding success including maturation time, fecundity, fertility, and sometimes, offspring viability (*19*, *20*), but the reproductive consequences of natural sex reversal in the wild are scarcely known. The only such empirical results come from a lizard species (*Pogona vitticeps*), in which gravidity was not observed in any of the three surveyed male-to-female sex-reversed individuals despite frequent gravidity among sex-concordant females (*21*). This aligns with the finding that sex-reversed females had lower fecundity than sex-concordant females in a captive-bred colony (*22*). In contrast, indirect evidence from two frog species suggests that some clutches are sired by female-to-male sex-reversed individuals (*18*, *23*, *24*).

Furthermore, it has been proposed that the offspring of sex-reversed individuals may be more sensitive to environmental stressors on the basis of observations that the progenies of sex-reversed individuals are more likely to undergo sex reversal than the progeny of sex-concordant individuals under the same environmental conditions

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(*25*). If such an effect exists, it counteracts the extinction-risk mitigating potential of the offspring of sex-reversed individuals; for example, the male surplus due to female-to-male sex reversal cannot be compensated by genetically female offspring if the latter also undergo sex reversal (*10*–*13*). Moreover, barely any information is available about the transgenerational effects of sex reversal on other aspects of stress tolerance, such as the ability to survive heat stress (*22*) or pathogen infection, even though these tolerances are highly relevant in the era of climate change and pandemic diseases (*26*–*29*). If sex-reversed individuals are "evolutionary dead-ends" as suggested by the above data on infertility and offspring vulnerability, then increasing sex-reversal rates driven by climate change may reduce the populations' ability to sustain themselves by producing enough offspring, which then may accelerate the ongoing mass extinction (*30*).

In this study, we combine empirical results from a series of experiments with multipopulation field data to weave a comprehensive picture of the relative reproductive performance of sex-reversed individuals in a population of agile frogs (*Rana dalmatina*). In this emerging model species for evolutionary-ecology research on sex reversal, ~20% of phenotypically male adults exhibit the female genotype (i.e., XX sex chromosomes instead of XY) across a number of wild populations, and the sex-reversal frequency increases with anthropogenic habitat modification (*24*). Experimental evidence indicates that this sex reversal is triggered by heat waves during larval development rather than by chemical pollutants (*31*–*34*). The spawning season lasts a few weeks in early spring, with males arriving earlier and staying longer at the spawning site, potentially mating multiple times (Fig. 1A), whereas females typically lay a single egg mass per season (*35*–*37*). When the density of males is high (like in our study population; ~400 males and ~200 females breeding in a ~180 m^2 pond), males switch from defending territories and calling for females stationarily to actively searching and fighting for females (*36*, *38*). Females clasped by an undesired male exert cryptic female choice by two strategies (*39*, *40*): First, they may delay oviposition, which can last up to 12 days (*41*), leaving time for other males to take over, and second, they may lay a smaller clutch. The latter allows them to reserve some of their eggs for later matings (*39*, *40*) or resorb the unreleased eggs to increase their clutch size for the next year (*42*). We leveraged this system to compare sex-reversed and sex-concordant males in terms of success in male-male competition, cryptic female choice, fertility, and offspring viability. To do this, we performed two sets of mating trials in a seminatural environment and then tracked offspring survival under heat stress and upon experimental infection with a viral pathogen, as follows.

First, we performed "fertility trials" to assess desirability to females and fertility by allowing 20 sex-reversed and 20 sex-concordant males each to mate singly with a gravid female in outdoor containers close to the breeding pond. The selected males all arrived on the same day to the spawning site at the start of the breeding season, and for each sex-reversed male, we chose a sex-concordant male counterpart with similar body mass. Thus, the two groups of males did not differ in arrival time and body mass, which are two major determinants of male reproductive success in anurans. After the fertility trials, we tested a subset of the males in "intrasexual competition trials" where one sex-reversed male and one sex-concordant male were allowed to compete for one gravid female. The two males competing in each trial were similar in size, and both had produced a clutch in the fertility trials. Meanwhile, in the clutches produced in the first trial, we monitored fertility and embryo survival. Then, to compare tolerance to environmental stress between the offspring of males with different genotypes, we kept a subset of tadpoles sired by 10 sex-reversed and 10 sex-concordant males and exposed them to a series of environmental stressors. First, we treated half of the tadpoles of each male with an experimental heat wave by raising the water temperature from 17° to 28°C for 6 days (*32*) and tested its effect on survival and sex reversal in the offspring. Second, after metamorphosis, we monitored the survival of the froglets for 3 months in seminatural outdoor enclosures, during which multiple heat waves occurred, making this summer the hottest and second-driest ever recorded in our country since 1901 (*43*, *44*). Last, we performed another experiment in which half of the froglets were exposed to ranavirus (Rv) infection, which can cause high mortality in amphibians (*45*).

Besides these experimental studies, we investigated a larger number of free-living animals to assess their reproductive potential. First, as male size may play a role in both intrasexual competition and mate choice, we measured the body mass of 113 males in our study population and combined these data with an earlier dataset of another 110 males collected across 11 breeding sites (*24*). Second, because sex-reversed males sire all-XX progeny, sampling the genotypic sex ratio of egg masses from the wild can provide information about the occurrence of reproduction by sex-reversed individuals in natural populations (*18*, *23*, *24*). Using this approach, we sampled 137 egg masses across 16 breeding sites over 5 years to estimate the proportion of clutches sired by sex-reversed males and to compare sex-reversal propensity between the offspring sired by sex-concordant and sex-reversed males.

Last, we used individual-based simulations to predict the consequences of our empirical findings for population dynamics under climate warming. We parameterized our earlier model (*10*) for agile frog life history, and we allowed for differences between sex-reversed and sex-concordant males in female choice and offspring viability on the basis of the results of the above experiments.

RESULTS

In the fertility trials, male genotypic sex had no significant effect on the latency from amplexus to spawning or on the total number of eggs laid (Table 1 and Fig. 2A). There was only one indication of reduced preference for some sex-reversed males: Three females paired with such males laid their eggs in two consecutive masses (Fig. 1B and fig. S1), while no such behavior was observed in females paired with sex-concordant males. This biologically large difference (27% versus 0%) was borderline significant because of the difficulty of estimating confidence intervals (CIs) for binomial data with complete separation (odds ratio: 10.29; 95% CI: 0.84 to 1454.03). In a follow-up experiment next year, we got similar results: Females laid "split clutches" for 3 of 6 (33%) sex-reversed and 1 of 14 (7%) sex-concordant males (odds ratio: 5.0; 95% CI: 0.52 to 66.88; note the wide CI due to near-complete separation). Putting the 2 years' data together, the difference was statistically significant (Table 1).

Because some of the males were exhausted near the end of the short spawning season, as indicated by releasing the female in the fertility trial without spawning or by not initiating amplexus in the next trial, we could perform eight competition trials. The female was clasped by one of the males in five competition trials, and it was the sex-reversed male that did so first in three of these five trials (60%). We observed no direct overtaking by competing males, but a sex-concordant male released the female after 24 hours of amplexus,

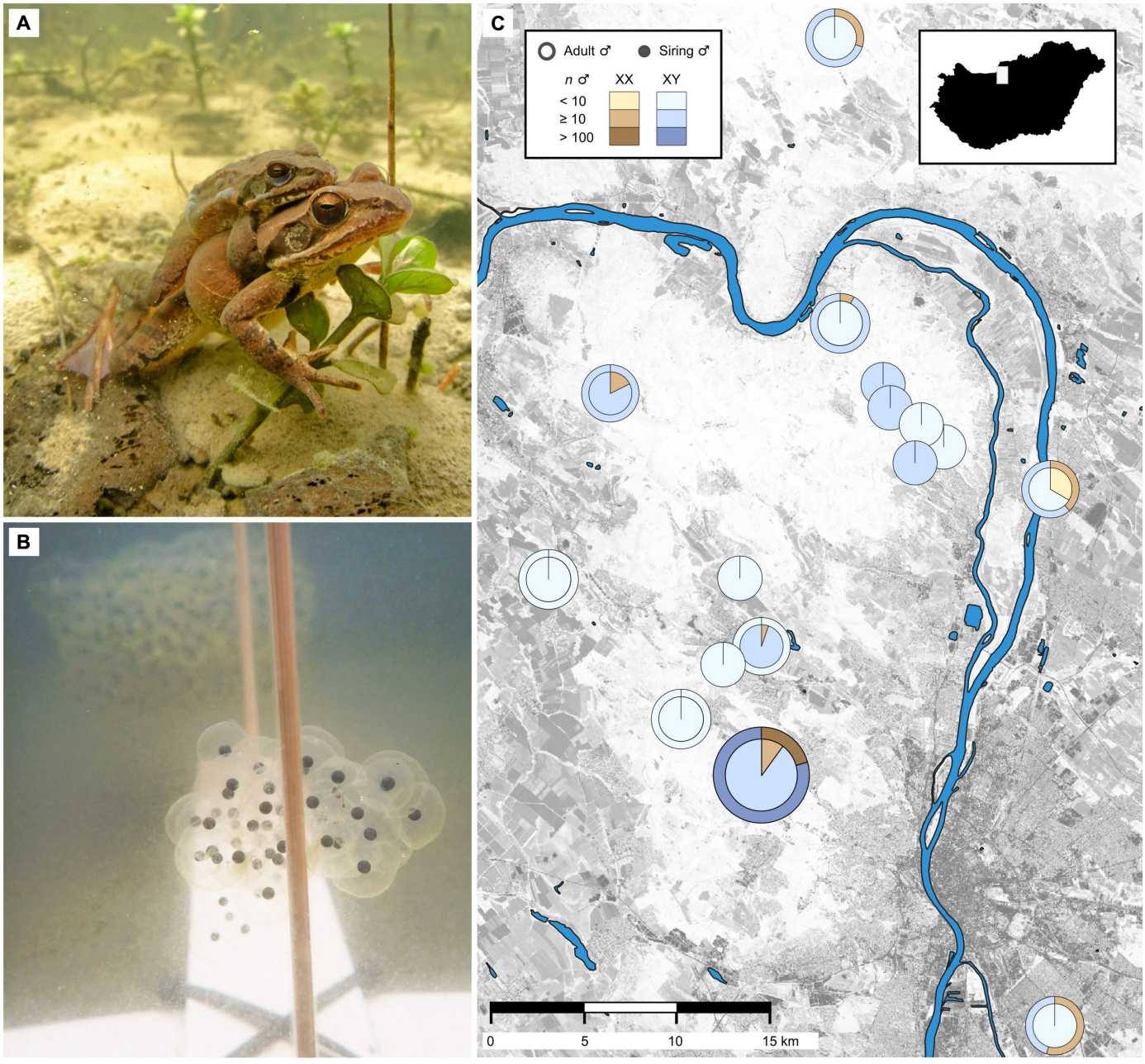

Fig. 1. Signs of cryptic female choice in agile frogs. (**A**) After engaging in mating amplexus, each female typically spawns a single egg mass per year. However, some females initially retain some of their eggs, which they may lay later. Photo by BIOSPHOTO / Alamy Stock Photo. (**B**) In our experiment, 27 % of females paired with a sex-reversed male spawned their eggs in two consecutive masses (fig. S1). (**C**) Across breeding ponds sampled in Hungary, the proportion of sex-reversed males was significantly lower among sires than among all phenotypic adult males captured in the spawning season (Table 1). Each circle represents a spawning site; the single large circle marks the population where the experiment was carried out. The outer ring represents all adult males captured at the site (a missing outer ring meaning no males captured), whereas the inner circle stands for sires inferred from the genotypic sex ratios of egg masses spawned in the wild. For rings as well as circles, the proportion of sex-reversed and sex-concordant males is shown in brownish and bluish colors, respectively, and the sample size is indicated by the shade of the color. The figure does not show three populations in which we had data on males' genotypic sex but not on clutch sex ratios; further details are given in the annotated R script (*48*).

after which the sex-reversed male took over. Three egg masses (all yielding viable embryos) were spawned in the competition trials, and the sex-reversed male was holding the ovipositing female in all these cases, while the sex-concordant male kept its distance. Latency to exhaustion across the two trials did not differ between sex-reversed and sex-concordant males (Fig. 2B).

In the fertility trials, females oviposited for 12 sex-concordant and 11 sex-reversed males; all these egg masses were fertile and yielded viable offspring. There was no difference between sex-reversed and sex-concordant males in the proportion of eggs that developed into embryos, the survival rate of embryos to tadpoles, and the growth and development rate of tadpoles (Table 1 and Fig. 2, C and D). Tadpole

Table 1. Details of the statistical models and the effect size of the sire being sex-reversed. Effect sizes at the data scale are shown with 95% CIs in brackets, and significant effect sizes are highlighted in bold font. Italic font indicates that the CI of the effect size includes no effect (difference = 0 or ratio = 1), but it is close to the significance threshold. The type of analysis is abbreviated as BINOM [generalized linear models with binomial or quasibinomial error and logit link (metric of effect size: odds ratio)], COX [Cox's proportional hazards models with or without random effects (metric of effect size: hazard ratio)], LM [linear models with or without random effects (metric of effect size: difference on data scale)], and NEGBIN [generalized linear models with negative-binomial error and log link (metric of effect size: incidence rate ratio)]. Treatment refers to the thermal treatment of tadpoles (control versus heated), and × stands for interaction. Random effects are written according to R syntax.

Dependent variable	Effect size (95% CI)	Type of analysis	R function	Main predictor	Additional predictors	Random effects	Unit of analysis	Sample size	Notes
Latency to spawning	0.85 (0.38, 1.94)	COX	coxme	Genotypic sex of the male	Male and female mass, female first or replacement	(1|male ID)	Females	57 females, laying eggs to 40 males	
Number of eggs	−36.45 (−239.55, 166.64)	LM	lm	Genotypic sex of the sire	Male and female mass, female first or replacement, amplexus date		Clutches	23 clutches by 23 sires	
Occurrence of split clutch	**7.48 (1.30, 78.92)**	BINOM	logistf	Genotypic sex of the sire			Clutches	43 clutches by 43 sires (23 from the main experiment and 20 from the follow-up study)	Due to quasi-separation in the data, Firth's bias reduction was used, without any additional predictor
Latency to exhaustion	0.59 (0.22, 1.60)	COX	coxph	Genotypic sex of the male	Male mass, time spent in amplexus, did it spawn in first phase		Males	40 males	
Proportion of fertile eggs	1.05 (0.25, 4.63)	BINOM	glm	Genotypic sex of the sire	Female first or replacement		Clutches	23 clutches by 23 sires	Quasibinomial error
Proportion of embryos surviving	0.83 (0.25, 2.68)	BINOM	glm	Genotypic sex of the sire	Female first or replacement		Clutches	23 clutches by 23 sires	Quasibinomial error
Tadpole body mass (g)	0.02 (−0.03, 0.06)	LM	lme	Genotypic sex of the sire	Tadpole age	(1|sibgroup/mesocosm)	Tadpoles	345 tadpoles by 20 sires, from 40 mesocosms	
Tadpole development (stage)	0.19 (−0.42, 0.79)	LM	lme	Genotypic sex of the sire	Tadpole age	(1|sibgroup/mesocosm)	Tadpoles	345 tadpoles by 20 sires, from 40 mesocosms	
Proportion of froglets surviving	0.84 (0.53, 1.35)	BINOM	glm	Genotypic sex of the sire	Treatment, treatment × sire sex		Enclosure	428 froglets from 8 enclosures	
Survival time after Rv exposure	1.23 (0.65, 2.34)	COX	coxph	Genotypic sex of the sire	Treatment, body mass			41 froglets	
Rv copy number	Control: 0.57 (0.17, 1.88); **heated: 4.38 (1.64, 11.67)**	NEGBIN	glm.nb	Genotypic sex of the sire	Treatment, treatment × sire sex, offset: liver mass		Froglets	37 froglets	

(*Continued*)

(*Continued*)

Dependent variable	Effect size (95% CI)	Type of analysis	R function	Main predictor	Additional predictors	Random effects	Unit of analysis	Sample size	Notes
Proportion of phenotypically female offspring	**Control: 4.86 (1.53, 15.44),** heated: 1.84 (0.67, 5.04)	LM	glm	Genotypic sex of the sire	Treatment, treatment × sire sex		Froglets	119 froglets	Overall effect regardless of tadpole treatment: **2.87 (1.37, 6.15)**
Offspring sex- reversal rate	*2.77 (0.88, 8.72)*	BINOM	logistf	Genotypic sex of the sire	Treatment		Froglets	92 XX froglets	Treatment × sire sex interaction cannot be tested due to separation
Male body mass (g)	−0.93 (−2.77, 0.92)	LM	gls	Genotypic sex of the male		varIdent: genotypic sex	Males	113 males from the main study population	Qualitatively same result with 223 males from 12 populations: 0.15 (−1.43, 1.74)
Proportion of sex- reversed individuals in the field	**5.57 (1.46, 21.3)**	BINOM	glmmPQL	Males vs sires		(1|pond)	Ponds	28 proportions from 19 ponds	Proportions calculated from 137 clutches with >5 offspring sexed from 16 ponds, and 295 males captured in 12 ponds
Offspring sex- reversal rate across populations	**7.58 (3.97, 14.5)**	BINOM	glmmTMB	Genotypic sex of the sire	Treatment	(1|pond) + (1|study)	Clutches	103 proportions from 16 ponds	Proportions calculated from 1,005 XX offspring raised from eggs in captivity

mortality was <1% regardless of thermal treatment. Similarly, froglet mortality did not vary with the sire's genotypic sex either upon exposure to natural heat waves (69% died overall; Table 1 and Fig. 2E) or to Rv (all infected froglets died within 12 days; Table 1 and Fig. 2E). However, among the froglets that had been exposed to heat stress during their larval life, Rv infection intensity was higher if the sire was sex-reversed rather than sex-concordant, whereas no such difference was observed among froglets that had not been exposed to heat as larvae (Table 1 and Fig. 3A).

As expected in an XX/XY sex-chromosome system, all froglets sired by sex-reversed males were genotypically female (XX), whereas 53.6% of froglets sired by sex-concordant males were genotypically male (XY). This resulted in a more female-biased phenotypic sex ratio among the offspring of sex-reversed sires compared to sex-concordant sires when the larvae had not been exposed to a heat wave (Table 1 and Fig. 3B, left panel). Some heat-unexposed offspring of sex-reversed sires were phenotypically males (Fig. 3, B and C, left panel), in agreement with earlier findings that sex reversal sometimes happens without heat exposure (*16*, *31*, *32*). Sex-reversal rates of XX offspring were increased by larval heat exposure (odds ratio: 3.52; 95% CI: 1.32 to 9.40; Fig. 3C), and regardless of larval thermal treatment, the offspring of sex-reversed sires were more likely to undergo sex reversal compared to the offspring of sex-concordant sires (Fig. 3C). This latter effect, although biologically large (15% versus 37%), was borderline significant because of the difficulty of estimating CIs for binomial data with near-complete separation (Table 1). This latter effect overwrote the effect of genotypic sex such that the phenotypic sex ratio did not differ between offspring of sex-reversed and sex-concordant sires when the larvae had been exposed to a heat wave (Table 1 and Fig. 3B, right panel).

The body mass of breeding males did not vary with genotype either in our main population (Fig. 2B, inset) or across multiple populations (Table 1). Among 137 egg masses across 16 breeding sites, we found 6 sibgroups in which the lack of XY offspring could not be attributed to sampling stochasticity. These six sibgroups were collected in three different years from four different populations >6 km from each other (Fig. 1C). If sex-reversed and sex-concordant males reproduce equally successfully in natural populations, the proportion of egg masses sired by sex-reversed males should be similar to the proportion of sex-reversed individuals among breeding males. Our data collected across wild populations clearly contradicted this, as the proportion of sex-reversed males (20%) was five times higher than the proportion of egg masses sired by sex-reversed males (4%; Fig. 1C). Furthermore, this multipopulation dataset significantly corroborated the trend that the experiment showed for increased sex-reversal propensity in the progeny of sex-reversed sires: In sibgroups

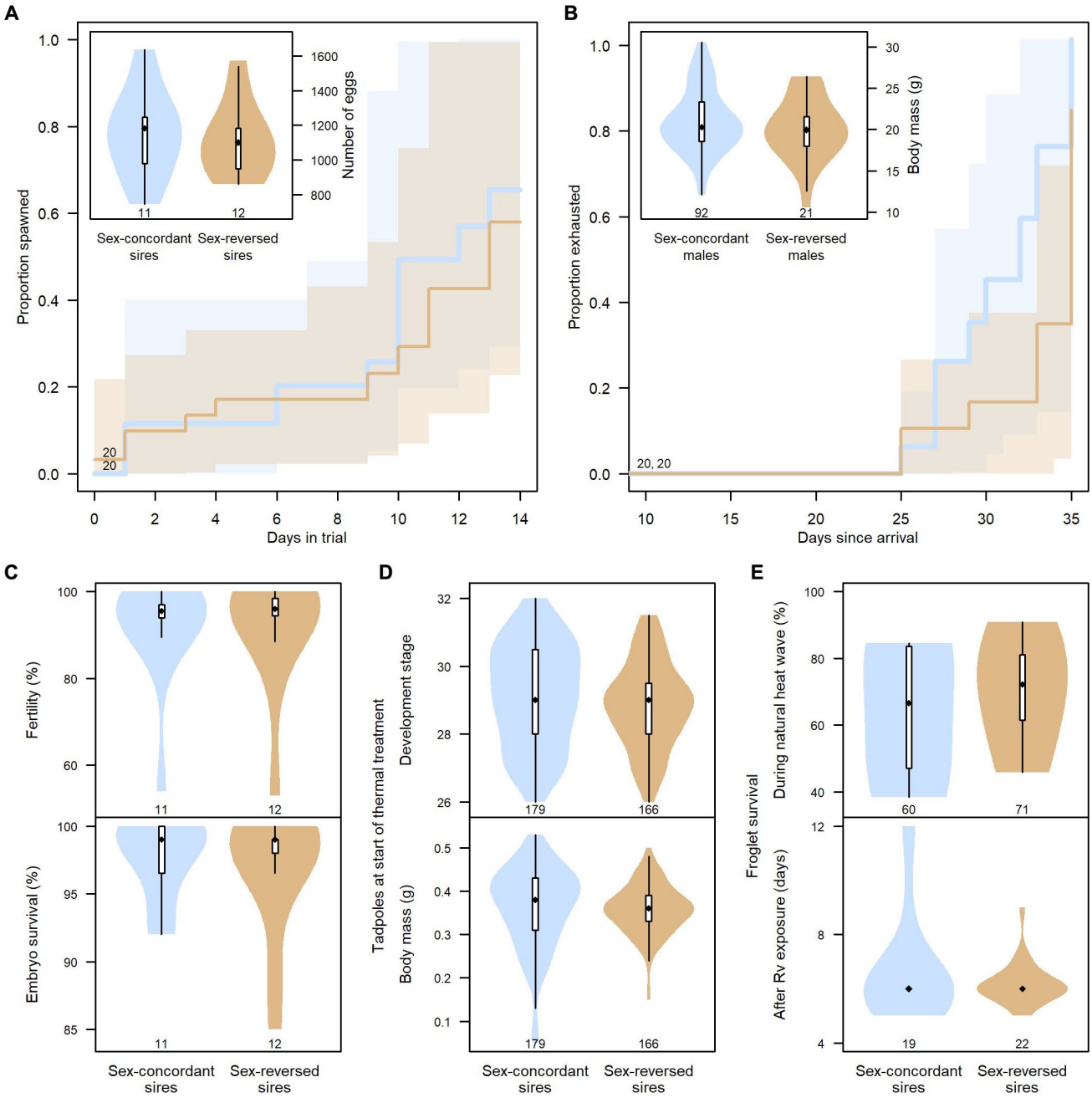

Fig. 2. Comparison of sex-reversed and sex-concordant males for measures of cryptic female choice, male vigor, and offspring viability. For spawning (A) and exhaustion [giving up amplexus; (B)], the step lines and the polygons illustrate the cumulative hazard and its 95% CI, respectively; the number at the start of each step line shows the number of participating males. In each violin plot (**A** to **E**), the black dot and the white box represent the median and interquartile range (IQR), respectively; whiskers extend to 1.5 × IQR; and the polygon is a kernel density plot. Sample sizes are shown under each plot. All comparisons between sex-reversed and sex-concordant males were statistically nonsignificant (Table 1).

collected from the field, the sex-reversal rate was higher for offspring of sex-reversed sires (61%; 95% CI: 44 to 76%) than for offspring of sex-concordant sires (17%; 95% CI: 12 to 23%; Table 1).

Modeling the consequences of these empirical findings under uninterrupted climate warming in small populations of frogs, the simulations predicted that some of the populations would already have started declining by our present time after 50 years of rapid climate warming, and all populations would die out within 60 to 70 years from now because of heat-driven sex reversal (Fig. 4). When we added Rv-induced offspring mortality that was higher for sex-reversed sires, it significantly advanced both the start of population decline (by 9.82 years on average; 95% CI: 6.04 to 13.60; Fig. 4) and extinction (by 9.54 years; 95% CI: 8.516 to 10.56; Fig. 4). Without Rv, females discriminating against sex-reversed males in mate choice

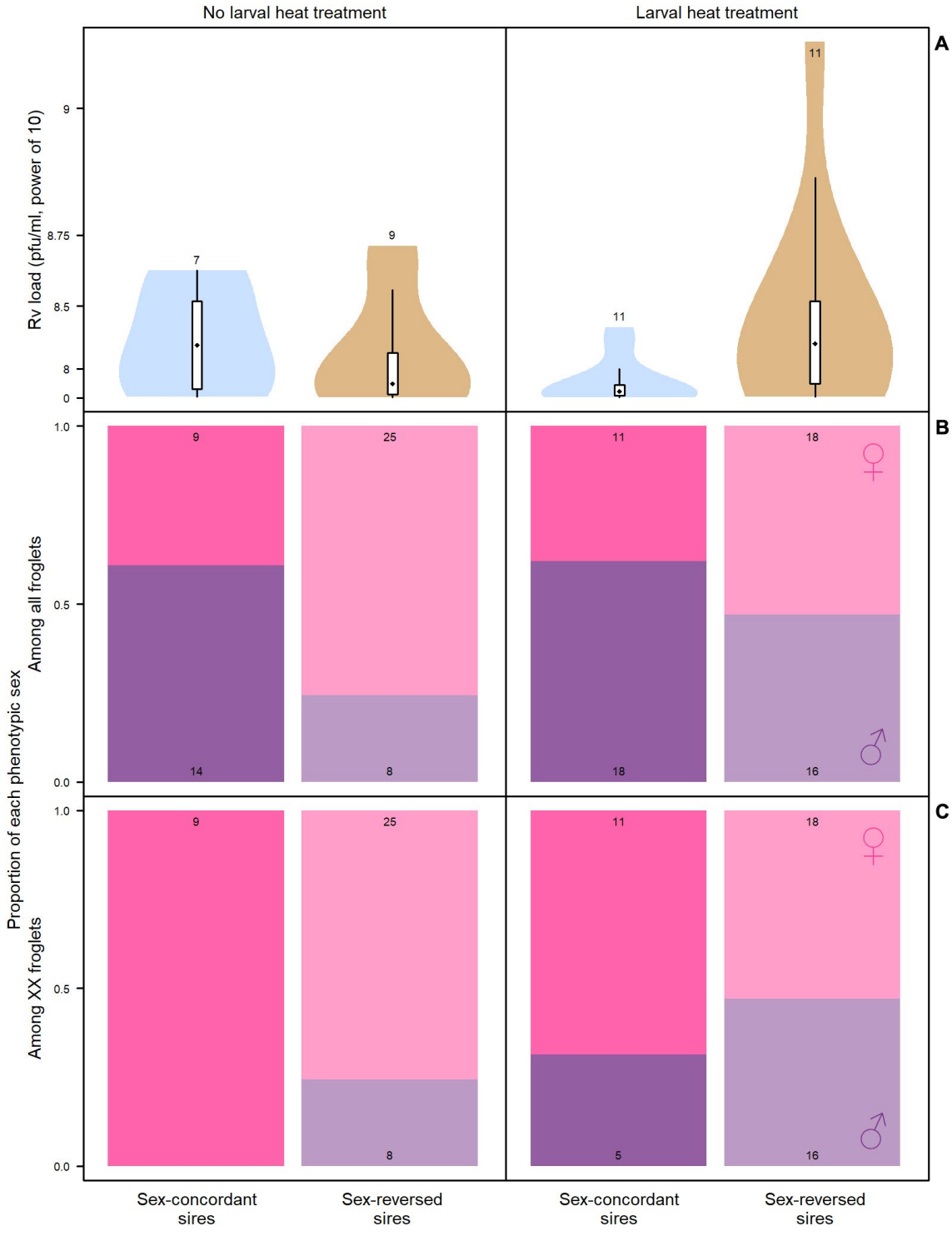

Fig. 3. Responses by offspring of sex-concordant and sex-reversed males to larval heat exposure and postmetamorphic Rv infection. Rv load (**A**) in offspring that underwent larval heat treatment was higher for sex-reversed sires than sex-concordant sires (Table 1), but there was no such difference without larval heat treatment (Table 1). Overall, the progeny of sex-reversed sires had more female-biased phenotypic sex ratio (**B**) and a tendency for a higher rate of XX-to-male sex reversal (**C**) compared to the progeny of sex-concordant sires (Table 1). Sample sizes are shown above (A) or within (B and C) each plot; see the caption in Fig. 2 for interpretation of violin plots.

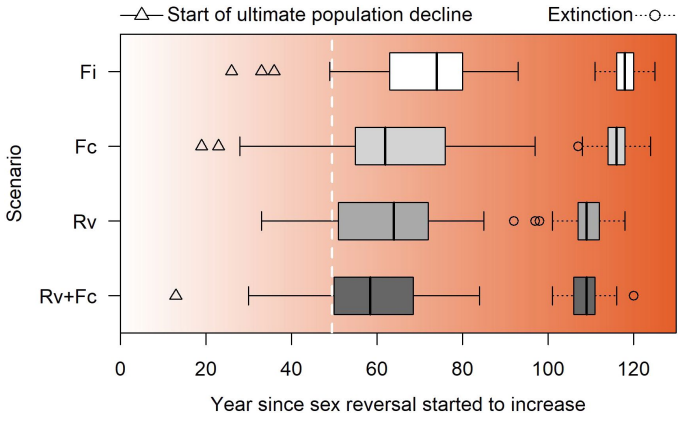

Fig. 4. Model-predicted timing of population decline and extinction depending on the relative success of sex-reversed males in passing on their genes to the next generation. In the abbreviations for scenarios, "Fi" stands for indiscriminate females, "Fc" for choosy females disfavoring sex-reversed males, and "Rv" for Rv-induced offspring mortality that is higher for sex-reversed sires. The start of ultimate population decline is the year in which effective population size permanently decreases below its average over 10 years before climate warming began to elevate the sex-reversal rate. In each box plot, the thick black line, box, whiskers, and empty symbols, respectively, show the median, IQR, IQR ± 1.5 × IQR, and data points more extreme than the latter from 100 simulations for each scenario. The white dashed vertical line marks the year in which the average frequency of sex-reversed males among adult males reaches its current level (20%) in agile frogs.

had a comparable hastening effect on population decline (by 8.21 years; 95% CI: 4.43 to 11.99; Fig. 4) and a smaller but still significant effect on extinction time (by 2.31 years; 95% CI: 1.29 to 3.33). In contrast, female choosiness had no significant effect in Rv-affected populations on either the start of decline (−3.19 years; 95% CI: −6.97 to 0.59; Fig. 4) or extinction (0.18 years; 95% CI: −0.84 to 1.20; Fig. 4).

DISCUSSION

In this study, we took a "total body of evidence" approach to combine multiple sources of evidence (within-population experiments, multipopulation observations, and theoretical modeling) to understand the consequences of sex reversal for reproductive success. Our unprecedented empirical dataset demonstrates that female-to-male sex-reversed agile frogs are not excluded from reproduction either by male-male competition or by female choice; they are fertile and produce viable offspring. As all their offspring are females genotypically, they have the potential to mitigate the shift toward male bias in the population sex ratio. However, this potential is compromised on several fronts, because sex-reversed males are less likely to become sires, and their offspring have a higher propensity for sex-reversal and may be more vulnerable to multiple stressors. Our theoretical model incorporating these effects predicts extinction of small populations in a few decades, with declines starting especially early in populations exposed to ranaviruses. Each of these findings is discussed in more detail below.

Regarding the first step of reproduction, that is securing a mate, we found several sources of evidence that sex-reversed males are capable in intrasexual competition. First, although our competition trials had very small sample size, they clearly showed that sex-reversed males can compete successfully against similarly sized sex-concordant males. Second, time to exhaustion also did not vary by genotypic sex in our experimental males. Third, a major determinant of male competitiveness, body mass did not differ by genotypic sex either in our main population or across multiple populations (*24*). Last, in several populations, we found egg masses in which all sampled offspring were genotypic females, showing that sex-reversed males can become sires even in breeding aggregations where males of all sizes are present. When viewed in isolation, each of these findings have their limitations, but they bolster each other to suggest that sex-reversed males are not necessarily inferior in intrasexual competition, which contrasts with a finding on captive-reared fish (*46*). Nevertheless, more data with larger sample sizes, preferably from natural breeding aggregations, will be needed to clarify the role of male-male competition in the reduced paternity rate of sex-reversed males we found in the wild.

Once the male has clasped a gravid female, the chances at successful reproduction may be similar between sex-reversed and sex-concordant males on the basis of the results of our fertility trials. However, the "split clutches" that we observed in these trials suggest that about one-third of females spawning with sex-reversed males used cryptic mate choice by laying only a portion of their eggs, whereas this almost never happened to sex-concordant males over 2 years of our experiments. This "split clutch strategy" occurs in agile frog females (*39*, *40*), and it likely serves to deceive the unpreferred male into terminating amplexus, which allows the female to lay the remaining eggs later for a more preferable male (*39*, *40*) or potentially to resorb the eggs for enhancing future fecundity (*42*). Apparently, this tactic did not work in our mating trials where no competing males and no other females were available, so the clasping males were either unwilling to release the females or clasped them again until they laid all their eggs. However, in the spawning aggregations of free-living agile frogs, the tactic of withholding some eggs may enable females to exercise their mating preferences despite male coercion.

If the seemingly lower attractiveness observed in our relatively small experimental sample of sex-reversed males also exists in breeding aggregations, this may explain our finding across a multitude of breeding populations that sex-reversed males sired a lower proportion of egg masses overall than expected on the basis of their relative frequency among phenotypic males. Although the proportion of egg masses containing XY offspring may increase by multiple paternity, the latter occurred too rarely in other populations (*38*, *47*) to account for the discrepancy we observed (*48*). Thus, the role of female choice in the reproductive success of sex-reversed males certainly deserves further research, especially given that theoretical models have already shown that female mating preferences can have profound effects on the population-level consequences of sex reversal under environmental change (*10*). The simulations we parameterized on the basis of our experimental results predicted that female choice against sex-reversed males would hasten the decline of effective population size roughly by a decade, which is a biologically large effect when small populations may not have more than a few decades left before going extinct because of rapid climate-driven masculinization. This highlights the fact that empirical studies like ours can help identify the most vulnerable species and populations. For example, in a study on common frogs (*Rana temporaria*), a species closely related to agile frogs, males with different sex-chromosome genotypes did not differ either in the probability of being found in amplexus rather than singly or in the proportion of clutches sired (although the authors attributed those genotypic differences to

polymorphisms in sex-chromosome differentiation rather than to environmental sex reversal) (*18*). Because the breeding season is even shorter in common frogs than in agile frogs, males of the former are very coercive and females can barely express their preferences (*39*, *40*), which might put this species at lower extinction risk (as in scenario "Fi" in Fig. 4).

After fertilization, our comprehensive data presented here demonstrate that offspring survival is unaffected by sire genotypic sex across multiple stressful periods during early life, including both experimental and natural heat waves and Rv infection. This aligns with the finding that thermal tolerance, measured as the critical thermal maximum, was not affected by maternal sex reversal in *P. vitticeps* lizards (*22*). However, in our study, viral load was higher in the progeny of sex-reversed sires compared to the offspring of sex-concordant sires when exposure to Rv occurred after larval heat stress. This suggests that these offspring have reduced tolerance when faced with a series of consecutive stressors. Although this difference in infection intensities did not translate to differences in survival time in our experiment, a lower viral load or slower disease progression might be lifesaving in more natural circumstances where the animals catch the virus from each other (*49*) or can move to thermally suitable microhabitats to bolster their immune defense and potentially cure themselves (*50*, *51*). Thus, our results highlight that the transgenerational effects of sex reversal on the vulnerability to multiple stressors are an important avenue for further experimental research. Notably, the taxa that are susceptible to environmental sex reversal include many species that are threatened by the contemporary spread of infectious diseases (*27*, *28*), which underscores the urgent need for a better understanding of the biodiversity consequences of synergistic anthropogenic environmental stressors.

As expected in an XX/XY sex-chromosome system, sex-reversed males had all-female offspring at the chromosomal level, whereas sex-concordant males had a 1:1 sex ratio in offspring genotypes. The all-XX offspring sired by sex-reversed individuals can be crucial for population viability in systems with female-to-male sex reversal, because these offspring can compensate for the surplus of phenotypic males caused by sex reversal and thus postpone the time of population extinction under climate warming (*10*, *11*). However, this protective effect may be diminished if the offspring of sex-reversed individuals are disproportionately sensitive to environmental stressors that increase either mortality or sex-reversal rates (*10*, *11*, *25*). Our findings suggest such a diminishing effect, because the offspring of sex-reversed sires were more likely to undergo sex reversal, and their phenotypic sex ratio was female-biased only in the heat-unexposed group but not in the heat-treated group. Furthermore, as the progeny of sex-reversed sires had higher Rv loads after larval heat exposure, this might further diminish their demographic contribution if it reduces survival in nature. When we assumed such an effect in the simulations, extinction time was advanced by a decade because of the extra mortality, and it did not make a difference if females disfavored sex-reversed males or not. This suggests that, from a conservation perspective, identifying pathogenic threats would be even more important than understanding mating dynamics for assessing population vulnerability to climate-driven masculinization.

Why should sex-reversed males be inferior in terms of paternity and offspring viability? One possibility is that they lack some Y-linked alleles. Although they look "normal" to the human eye and have similar body mass to sex-concordant males, it would be worth finding out if they differ in more difficult-to-detect aspects such as acoustic calls or sex pheromones, which play a role in mating (*35*, *52*). Genes related to immunity might also be sex-linked and epigenetically transmitted to offspring (*53*), which is another interesting avenue for future research as it is especially unexplored in nonmodel animals. However, because most species with sex reversal have homomorphic sex chromosomes (*6*), suggesting relatively limited genetic differentiation, it seems more plausible that the reproductive differences between sex-reversed and sex-concordant males might not lie in their genotypes. Instead, the environmental conditions that trigger sex reversal may exert other long-lasting effects, too. For example, early-life exposure to heat stress not only causes sex reversal but also has various negative effects on physiological and life-history traits (*24*, *31*, *32*). While some of these effects might result in disadvantages when it comes to breeding, this does not necessarily mean that sex reversal itself is disadvantageous to fitness. To the contrary, it is hypothesized that sex reversal is the "best out of a bad job," whereby individuals handicapped by their early environment can switch to the phenotypic sex that is less disadvantaged than the other (*16*). This idea has received very little empirical and no explicit theoretical testing yet, but if it proves true, it might mean that the future is not as dark as what the models predicted so far. Incorporating such intricate effects of sex reversal into theoretical models will improve our understanding of its consequences and will help identify the populations that are the most in need of protection against climatic and pathogenic threats.

MATERIALS AND METHODS

Experimental procedures

The study was approved by the local Ethics Committee of the Plant Protection Institute and licensed by the Environment Protection and Nature Conservation Department of the Pest County Bureau of the Hungarian Government (PE-06/KTF/07949-6/2023 and PE/EA/00270-6/2023). On 6 February 2024, we erected a drift fence with pitfall traps around our study pond (47.551195°N, 18.926682°E). On 12 February, we captured 126 males (the first 27 males arriving on 10 and 11 February and the 271 males arriving later were not used in this study). We took a buccal swab sample from each male, housed the animals overnight individually in 3-liter plastic boxes lined with wet paper towels, and released them the next morning into containers (87 by 64 cm) that were placed next to the pond, filled with pond water to a ~15-cm depth. Each male was individually marked with a small piece of embroidery thread tied to its knee such that it would not harm the animal but would not fall off, with each male within each container receiving a thread of different color. We kept up to 10 males in each container until they were either used in the experiment or released into the pond. From the swab samples, we extracted DNA and identified genotypic sex using our established protocol (*24*). This yielded 21 sex-reversed and 92 sex-concordant males [for 13 males, the results were inconclusive (*48*)]. Of these 113 individuals, we selected 20 duos each consisting of one sex-reversed male and one sex-concordant male such that the two males within each duo would be similar in body mass (difference $\leq$1.5 g; median: 0.095; mean $\pm$ SD: 0.26 $\pm$ 0.35; body mass varied between 10.59 and 26.39 g). For the 40 selected males, genotypic sex was further confirmed by Sanger sequencing on the Rds3 marker and, additionally, on the Rds1 marker for four males whose polymerase chain reaction results for this marker were ambiguous. The remaining 86 males were released from the containers into the pond on 23 February.

The experiment comprised two trials. In the first trial, each male was allowed to mate with a single female to assess their fertility, the male's attractiveness to the female, and the viability of their offspring. In the second trial, we offered each male duo one female to measure the males' success in intrasexual competition. The logic of trial order was that we wanted to compare fertility between sex-reversed and sex-concordant males under the same conditions, which would not have been possible in the competition trials where only one of the two males could have spawned; also, we wanted to avoid losing males for the fertility trials because of exhaustion.

For the first trial, we used similar containers to those in which the males were kept until the start of the experiment. These containers were placed next to the pond, so the animals in the containers could hear the mating calls of the frogs in the pond surrounded by their natural woodland environment. For the second trial, we placed 72-cm-diameter containers in a fenced area ~400 m away from the pond, where we could protect the cameras from theft. Above each container, there was a horizontal metal rod on which we could mount a trail camera. We filled each container to a ~15-cm depth with tap water 1 week before starting this phase to let the chlorine evaporate. In both kinds of containers (i.e., first and second trials), to offer a substrate to which the female can attach the egg mass, we placed three wooden sticks that were fixed to a tile with coral glue to keep them underwater (Fig. 1B).

Males in the 20 duos entered the experiment as follows. Whenever two gravid females were captured at the drift fence on the same day, we assigned them randomly to the two males of a randomly picked duo. We measured the body mass of each animal and released one male and one female into a container. The two members of each male duo were always placed in two containers next to each other and at the same time. Every morning, we checked whether the male and female were in amplexus and whether they had spawned. If a male did not initiate amplexus for 48 hours after starting the experiment ($n = 1$) or if the pair did not spawn for 2 weeks ($n = 21$), the male was provided with another gravid female as a replacement (except one male for which the season was too late for capturing a gravid female for replacement). The first trial ended when eggs had been laid and the male released the female ($n = 23$), when the replacement female did not lay eggs for 7 days ($n = 9$), or when the male became exhausted, as indicated by releasing the female after several days of amplexus ($n = 3$; two of those were replacement females, as only the male with the smallest body mass gave up on his first female) or by not initiating amplexus with the replacement female for 24 hours ($n = 4$). Note that agile frogs migrate to the spawning sites after winter hibernation without feeding and typically spend 2 to 3 weeks there (*54*), which is somewhat shorter than the maximum duration of the first trial of our experiment (starting on 23 February and ending on 18 March, 1 day after the last females arrived to the pond), likely explaining why some males lost their libido and/or stamina by the end of the first trial. It is very unlikely that males gave up because of disturbance, given that disturbance in our study was minimal (one or two brief visits daily to check for the presence of amplexus and eggs) and constant over time, yet no male gave up before 25 days postarrival. Reproductive exhaustion is known to occur in male agile frogs and other species with short breeding seasons (*40*, *55*), as they lose considerable body mass (up to 17% in our experiment) over the season (*56*) during which they rarely feed (*57*).

After the first trial, we measured the body mass of the frogs again and released the females into the pond if they still had eggs or in the woods if they had spawned. If the male did not show signs of exhaustion, and gravid females were still available, we kept the male in a container similar to the first-trial containers but close to the second-trial containers until the other member of the duo finished his first trial. The next day, we released both males with a gravid female into a second-trial container and mounted a trail camera above it that recorded a picture every 5 min. Each male was fitted with an embroidery thread around its waist, which held a small piece of rubber tape above its back that was either white or black-and-white striped, ensuring individual identification both in the color pictures taken in daylight and in the grayscale images taken during the night. The second trial ended when eggs had been laid and the male released the female ($n = 3$), when the female did not lay eggs for 10 days ($n = 1$), or when the males became exhausted ($n = 4$). After the second trial, we released the three frogs into the pond. For 12 duos, the second trial was not initiated because of male exhaustion or lack of gravid females; these males were also released into the pond.

The 23 egg masses laid in the first trial were left undisturbed for a week in their containers to see if the embryos started to develop. After 1 week, we gently detached the egg mass from the wooden stick and measured its weight by transferring it with a dipnet into a smaller container holding spring water. Then, we removed five or six small clumps from the clutch (sampling both egg masses if the female deposited her eggs in two masses), totaling 100 to 132 eggs (mean ± SD: 105.65 ± 7.70), and for each small clump, we measured its weight (±0.1 g) and counted the number of eggs in it (mean ± SD: 20.58 ± 13.45). We calculated the mean egg weight for each small clump and divided the weight of the entire clutch by its mean egg weight to estimate the total number of eggs laid by the female. Then, we placed the remaining egg mass into the shallow, vegetated part of the pond. The small egg clumps used for measuring egg weight were placed back into the container, enclosed in a 28 by 34–cm fruit bag that was kept dilated by two plastic rings, to prevent hatchlings from escaping but allowing free water and gas exchange and colonization by periphyton. When the offspring developed into free-swimming tadpoles, i.e., Gosner's (*58*) developmental stage 25, we counted the number of living tadpoles, dead embryos and tadpoles, and the eggs that did not develop into embryos. The three egg masses laid in the second trial were kept in their containers until they were close to hatching to ascertain whether the embryos were viable and then released into the pond without any measurement.

After counting the first-trial offspring at developmental stage 25, we kept 40 healthy-looking tadpoles from each of 10 sex-reversed sires and 10 sex-concordant sires. The rest of the tadpoles were released into the pond, including all tadpoles from the remaining three egg masses: the earliest-spawned, the latest-spawned, and one with the lowest viability and high deformity of offspring (the latter egg mass is shown in Fig. 1B). The 40 tadpoles retained from each egg mass were placed into two outdoor mesocosms (20 tadpoles per mesocosm), where they were raised for at least 2 weeks (14 to 20 days; mean ± SD: 16.15 ± 2.35). The mesocosms were set up by filling 42 by 72 by 30–cm plastic tanks with 65 liters of tap water, adding 1 liter of pond water and 40 g of dried beech (*Fagus sylvatica*) leaves 3 days later, covering them with mosquito-net lids, and allowing for a self-sustaining ecosystem to develop for at least a week before introducing the tadpoles. When the youngest tadpoles reached 2 weeks of age, in each mesocosm, we counted the number of surviving tadpoles and selected 12 healthy-looking individuals for further experiments. The remaining tadpoles (up to eight per mesocosm)

were weighed, examined for development stage, and released into the pond. All tadpoles were in similar developmental stages at this time (stages 26 to 32), corresponding to small limb buds with no toe differentiation yet.

The retained tadpoles were exposed to a 6-day heat wave to assess their heat tolerance following our methods described earlier (*16*, *32*). In short, each tadpole was housed individually indoors in a plastic box containing 1.7 liters of ultraviolet-sterilized, fully aerated reconstituted soft water (RSW), and lighting was set to follow the natural photoperiod. In each group of 24 siblings (henceforth, sibgroups), 12 tadpoles were kept at control temperature (mean ± SD: 17.31 ± 0.12°C), whereas for 12 tadpoles, the water was gradually heated up to treatment temperature (mean ± SD: 27.66 ± 0.41°C) and kept there for 6 days. Every other day, we changed the rearing water and fed the tadpoles with chopped, slightly boiled spinach. At the end of the 6 days, we stopped the heating and allowed the water to cool down to control temperature. The next day, we moved the tadpoles back into the outdoor mesocosms, each container housing 12 siblings that had received the same treatment. Twenty-four days after ending the treatment, when some of the tadpoles reached metamorphic climax (stage 42: appearance of forelimbs), we moved all animals to eight new mesocosms such that each mesocosm housed up to 60 animals that originated from the same type of sire (sex-reversed or sex-concordant) and underwent the same treatment (control or heated). These mesocosms were 30-cm-high, 80-cm-diameter containers that we filled to the brim with pond water and added branches and small cork rafts to help the metamorphs leave the water. Each mesocosm was dug into the soil within a 3 by 3–m enclosure made of mosquito net that was 60 cm high above ground and dug 20 cm deep into the ground. To keep predators away, we removed large ground beetles by trapping and covered the top of the enclosures with 17-mm chicken wire. The enclosures were shaded by trees and had natural vegetation, but we complemented the naturally available food resources with small crickets (*Acheta domesticus*) once or twice a week. Upon each visit, we checked the enclosures from the outside to search for dead froglets; these checks did not recover corpses while the weather was favorable. However, our region was hit by record-breaking hot and dry weather in July and August; during this time, 43 froglets apparently died from heat stress, as they were found dead in or close to the water in good body condition with no sign of injury. We dissected these corpses to identify phenotypic sex by gonad morphology and stored a tissue sample (hind feet) for genotypic sexing in 96% ethanol.

On 27 August, we collected all surviving froglets ($n = 88$) from the enclosures and brought them indoors into a quarantine lab where air temperature was 23.66 ± 0.40°C and lighting was set to follow the natural photoperiod. We measured their body mass and took buccal swab samples, which verified that they were negative to Rv. We housed each froglet individually in a 2-liter plastic box covered with a transparent, perforated lid, lined with wet paper towels and a piece of egg carton as shelter. The next day, we exposed each froglet to a 5-hour treatment in a 6-cm-diameter petri dish filled with 8 ml of RSW, following earlier methods (*59*). We exposed half of the froglets recaptured from each enclosure to Rv virions (Frog Virus 3; American Type Culture Collection no. VR-567) at a concentration of 3×10^6 plaque-forming units (pfu)/ml, while the other half received sham treatment (the nutrient medium supplemented with 2% fetal bovine serum without the virus). We applied a stratified random sampling design to ensure that the two treatment groups did not differ by body mass, sire genotypic sex, and larval heat treatment (table S1). We checked mortality every day for 2 weeks. Twice a week, we fed the froglets ad libitum with small crickets and sprinkled their boxes with RSW to maintain humidity. When an individual was found dead, we dissected it to identify phenotypic sex by gonad morphology, and we stored a tissue sample (hind feet) for genotypic sexing in 96% ethanol. For measuring Rv infection intensity, we stored two liver lobes in 96% ethanol and measured their mass. The froglets that survived for 2 weeks were dissected the same way after euthanasia in a shallow water bath of tricaine-methanesulfonate (5 g/liter; MS-222) buffered to neutral pH with the same amount of disodium hydrogen phosphate. From the tissue samples of froglets, we identified genotypic sex with the methods used for adult males (*24*). From the liver samples and buccal swabs, we measured Rv infection intensity with quantitative polymerase chain reaction (*59*, *60*). Liver samples were negative to Rv for all unexposed froglets, except for one individual with a very low Frog Virus 3 copy number (1174 pfu/ml) probably due to contamination during dissection.

Follow-up experiment

In the 2025 spawning season, we repeated the mating trials for the purposes of another experiment where sex reversal was not the primary question (not yet published). The methods were similar to the fertility trials of 2024, but genotypic sex was not tested before the trials, and therefore, the sample was not balanced for comparing similarly sized sex-concordant and sex-reversed males. We used the occurrence of "split clutches" from this experiment to complement our 2024 data. This resulted in twice as large sample size and removed the problem of complete separation in the binomial data (see the "Statistical analysis" section).

Field sampling

To assess reproductive potential in a larger number of free-living sex-reversed males, we created two datasets. First, we took our earlier body-mass measurements collected across 11 populations (Fig. 1C) from 21 sex-reversed and 89 sex-concordant males (*24*) and combined them with the data of the 21 sex-reversed and 92 sex-concordant males weighed in the present study upon arrival to the breeding pond.

Second, we compiled the data we had collected for other studies in 2018 to 2023 on the genotypic sex of siblings from 147 egg masses across 16 populations (*16*, *31*, *32*, *61*–*63*). In these samples, genotypic sex was identified after several months of raising the animals from eggs in captivity. To test whether these sibgroups originated from sex-reversed sires, we performed a simulation in which we sampled a Bernoulli distribution with a probability of 0.5, representing the proportion of offspring that should inherit the Y chromosome if the sire is sex-concordant. For the sample size of each sibgroup in our dataset, we ran 10,000 iterations and calculated the 99% CI of the proportion of XY offspring. Whenever this CI excluded zero, meaning <1% chance of getting all-XX offspring merely by random sampling from a sex-concordant sire, we concluded that the sibgroup originated from a sex-reversed sire. We used this approach to calculate the proportion of egg masses sired by sex-reversed males for each population, and we compared this to the proportion of sex-reversed males among phenotypic males captured in the 12 populations that we sampled as described above for body mass. These two proportions are expected to be similar if sex-reversed and sex-concordant males are equally successful at breeding in nature. Because the simulation showed that the minimally required sample size for identifying a sex-reversed sire with 95% confidence was six offspring, we excluded the

sibgroups with $n \leq 5$ from further analyses, leaving 137 sibgroups with 16.3 ± 10.4 (mean ± SD; up to 52) siblings in each.

We used the same multiyear dataset to test whether offspring sex-reversal rates differ between sex-reversed and sex-concordant sires across populations. To this end, we inferred sire genotype from the presence/absence of XY offspring, as described above. Of the 137 sibgroups for which this inference was possible, we had data for 103 sibgroups on the phenotypic sex of the offspring ($n = 1005$ froglets in total). These data came from six studies in 5 years, representing 16 populations. In each study, the offspring were collected as eggs and raised in the laboratory under different larval treatments: heat, chemicals, or both (*16*, *31*, *32*, *61*–*63*). For each sibgroup, we calculated the proportion of phenotypic males among genotypic females as the rate of sex reversal.

Statistical analysis

Statistical analyses and sample sizes are summarized in Table 1. Detailed description of our statistical analyses, with R (*64*) scripts and outputs, is available along with the datasets in the FigShare repository (*48*).

We calculated the latency to spawning as the number of days from the initiation of amplexus to egg laying and the latency of exhaustion as the number of days from capture to exhaustion (defining exhaustion as the male releasing the female without spawning or not initiating amplexus either with the replacement female in the first trial or with the female in the second trial). We analyzed these data with Cox's proportional hazards models, treating cases where spawning or exhaustion did not happen as censored observations, and we expressed the effect sizes as hazard ratios. For spawning latency, when the male was given a replacement female, the latencies of both females were used as repeated measures.

For proportions and presence/absence data, we used generalized linear models with binomial (or quasibinomial, depending on overdispersion) error and logit link, and we expressed the effect sizes as odds ratios. In particular, these models were used for the presence of a second egg mass ("split clutch" in the first trial), fertility rate estimated as the proportion of eggs that developed into embryos, embryo viability rate estimated as the proportion of embryos that reached stage 25 alive, the proportion of froglets surviving in each enclosure, and the proportion of phenotypically female froglets out of all froglets (i.e., phenotypic sex ratio) and out of XX froglets (i.e., sex-reversal rate). In binomial models, we applied Firth's bias reduction method to deal with separation in the presence of "split clutch" and in offspring sex-reversal rates to mitigate estimation uncertainty, which is high when variance is zero or near-zero in at least one group (i.e., no "split clutches" for sex-concordant sires; no sex reversal in offspring of sex-concordant sires if they had not been heat treated as larvae).

To compare the proportion of sex-reversed males between siring males (as estimated from clutch sex ratios) and all males captured in wild populations, we used a generalized linear mixed model with quasibinomial error, and we included population as a random factor. This design adequately handles the heterogeneity in our multiyear data by weighting for differences in available sample sizes and allowing for spatial variation in sex-reversal frequency by treating males and sires (clutches) as repeated data. Note that the two multipopulation datasets overlap, but we do not have data on breeding males in 7 and on clutches in 3 of the total of 19 breeding sites; this is appropriately dealt with in the applied mixed model. To compare sex-reversal rate between the lab-raised offspring of sex-reversed and sex-concordant sires inferred from the multipopulation dataset, we used a generalized linear mixed model with binomial error and two crossed random factors: population identifier and study identifier.

We used a simple linear model to analyze the number of eggs spawned, linear models with mixed effects (mesocosm nested in sibgroup as random factors) to analyze tadpole size and developmental stage, and a generalized least-squares model to compare the body mass of sex-concordant and sex-reversed males captured for this study. In the latter model, we allowed for different within-group variances for the two types of males. Last, we used a generalized linear model with negative binomial error and logit link to analyze Rv infection intensity, and we expressed the effect sizes as incidence rate ratios.

In all statistical models, the predictor variable was sire genotypic sex, along with potentially confounding variables such as sire body mass, date, or offspring treatment, whenever relevant (Table 1); for further details, see our annotated R code (*48*). Because the addition of potentially confounding variables never changed the effect of sire genotypic sex qualitatively, in Table 1, we present the simpler models that included the main predictor only; the full results of more complex models can be found in our annotated R script (*48*). For each analysis, we checked that the statistical requirements were met by plotting residual diagnostics (*65*). From every statistical model, we estimated the 95% CI of the effect sizes to make inferences about significance (*66*–*68*). For cases where the zero effect (odds ratio = 1) falls within the CI but very closely to the significance threshold, we use the term "borderline significant."

Theoretical modeling

We used the individual-based simulations developed in our earlier study (*10*) to model changes in demography and population genetics, assuming a constant rise in the frequency of sex reversal resulting from contemporary climate warming. All parameters were set as in the previous study, except for the following, as explained in more detail in table S2. On the basis of the literature on agile frog life history, we set the annual survival rate in juveniles to 0.3 (*69*), age of maturity to 2 years in males and 3 years in females (*69*, *70*), and maximum life span to 10 years (*71*). Sex-specific adult survival rates are unknown in this species, although return rates between 0.4 and 0.6 were reported (*71*). Therefore, we assumed 0.5 annual survival for both sexes, resulting in a realistic adult sex ratio of ~78% males (*36*, *71*), which is close to 72% in our main study population. As the average clutch size is ~1000 eggs (*72*) and the maximum survival from hatching to metamorphosis is 27% (*73*), we set maximum offspring production per female to 270. We set the environmental carrying capacity to 6000 offspring to model a small breeding population of about 200 adults (*74*). These parameters were fixed in all simulations. We ran 100 simulations each for the following scenarios. In scenario "Fi" ("Females indiscriminate"), sex-reversed and sex-concordant males do not differ either in the likelihood of females choosing them or in offspring viability. In scenario "Fc" ("Females choosy"), we assume that all females have a single preference allele that encodes 0.27 probability of rejecting sex-reversed males (0.73 probability of preferring concordant males) on the basis of our finding in the current experiment (see Results). In scenario Rv ("Ranavirus"), we assume that the population coexists with Rv, and this decreases juvenile survival to a greater extent for sex-reversed sires (0.45) than for sex-concordant sires (0.75) on the basis of the mortality rates observed in a related anuran species (*49*). In scenario "Rv + Fc," we combined

scenarios Rv and Fc. From each run, we extracted several demographic metrics the same way as in our earlier study (*10*). We used linear models to compare the scenarios for the timing of ultimate population decline (i.e., the year in which effective population size permanently decreased below its average over 10 years before the start of climate warming when the population was in a stable state) and extinction time (i.e., the number of years the population survived after the start of climate warming). For every pairwise comparison between scenarios, we estimated the 95% CI of the effect sizes to make inferences about significance (*66*–*68*).

Supplementary Materials

This PDF file includes:

Fig. S1
Tables S1 and S2
References

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