Complex effects of sex reversal on reproductive success in wild frogs

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Sex reversal, when the environment overrides genotypic sex determination, is theorized to exert wide-ranging effects on population dynamics and evolution in ectothermic animals [1–6]. The expected outcomes critically depend on the reproductive ability of sex-reversed individuals and the viability of their offspring, but empirically next to nothing is known about these traits in natural populations. Here, we address this knowledge gap with the first comprehensive dataset using the agile frog, an amphibian in which larval heat stress causes female-to-male sex reversal [7,8]. In a wild population where 20% of adult males are genotypic females, we compare sex-reversed and sex-concordant males for fertility, attractiveness to females, intrasexual competitiveness, and offspring survival under benign conditions, heat stress, and viral infection. We demonstrate that sex-reversed males exhibit uncompromised potential to mate and produce viable offspring, which are all genotypic females with female-biased phenotypic sex ratios. However, clutch sex ratios sampled across wild populations reveal reduced paternity by sex-reversed males, and our experiment indicates cryptic female choice as the mechanism. Furthermore, the offspring of sex-reversed sires have higher ranavirus loads after larval heat exposure, suggesting increased vulnerability to multiple stressors. These effects of sex reversal predict complex consequences of environmental change for the fate of biodiversity.

After discovering half a century ago that sex is determined by environmental factors in some reptiles and fish [9,10], the advancement of genomic techniques over the last two decades allowed us to realize that the natural environment can influence sex also in species that have genotypic sex determination [5,11]. This environmental override results in a mismatch between phenotypic and genotypic sex, a peculiar phenomenon termed sex reversal. A vast body of laboratory experiments show that many species are susceptible to sex reversal induced by

environmental temperatures and chemicals [12–14], and sex-reversed individuals have been found in nature whenever they were looked for in published studies of wild populations of amphibians, reptiles, and fish [12,15,16]. Theoretical models predict that thermal sex reversal has wide-ranging consequences for population dynamics, microevolution, and phylogeography, particularly under persistent climatic warming [1–6]. 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 [1–4]. 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 [17]), this issue is of particular importance for biodiversity conservation.

Empirically, however, only a tiny fraction of extant species have been studied for the evolutionary ecology of sex reversal [16,18], 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 [19,20]. 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 [21,22], 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 [23]. This aligns with the finding that sex-reversed females had lower fecundity than sex-concordant females in a captive-bred colony [24]. In contrast, indirect evidence from two frog species suggests that some clutches are sired by female-to-male sex-reversed individuals [20,25,26].

Furthermore, it has been proposed that the offspring of sex-reversed individuals may be more sensitive to environmental stressors, based on observations that the progeny of sex-reversed individuals are more likely to undergo sex reversal than the progeny of sex-concordant individuals under the same environmental conditions [27]. However, 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 [24] or pathogen infection, even though these tolerances are highly relevant in the era of climate change and pandemic diseases [28–31]. If sex-reversed individuals are indeed "evolutionary dead-ends" as suggested by the above data on infertility and offspring vulnerability, then increasing sex-reversal rates driven by climate change may accelerate the ongoing mass extinction [32].

In this study, we combine empirical results from a series of experiments with multi-population 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, ca. 20% of phenotypically male adults exhibit the female genotype (i.e. XX sex chromosomes instead of XY) across a number of wild populations, and sex-reversal frequency increases with anthropogenic habitat modification [26]. Experimental evidence indicates that this sex reversal is triggered by heat waves during larval development rather than by chemical pollutants [7,8,33,34]. 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; ca. 400 males and ca. 200 females breeding in a ca. 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 (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 or resorb the unreleased eggs to increase their clutch size for 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 semi-natural environment and then tracked offspring survival under heat stress and upon experimental infection with a viral pathogen, as follows.

First, we performed 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. In these trials, male genotypic sex had no significant effect on the latency from amplexus to spawning or on the total number of eggs laid (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, Extended Data Fig.

1), while no such behavior was observed in females paired with sex-concordant males. This suggests that some females clasped by sex-reversed males employed cryptic mate choice [39] by laying only a portion of their eggs to deceive the unpreferred male into terminating amplexus, which may allow the female to lay the remaining eggs later for a more preferable male or to resorb them for enhancing future fecundity. 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.

After the fertility trials, we tested a subset of the males in intrasexual competition trials where one sex-reversed 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. 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 competition trial, we could perform 8 competition trials. The female was clasped by one of the males in 5 trials, and it was the sex-reversed male that did so first in 3 of these 5 trials (60%). We observed no direct overtaking by competing males, but a sex-concordant male released the female after 24 hours of amplexus, 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 (Fig. 2cd). 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 °C to 28 °C for 6 days [8]. Second, after metamorphosis, we monitored the survival of the froglets for three months in semi-natural 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]. Finally, we performed another experiment in which half of the froglets were exposed to ranavirus (Rv) infection, which can cause high mortality in amphibians [45]. Tadpole 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 or to Rv (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 (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 (odds ratio ± SE = 4.86 ± 2.87, p = 0.015; Fig. 3b). Some heat-unexposed offspring of sex-reversed sires were phenotypically males (Fig. 3b) in agreement with earlier findings that sex reversal sometimes happens without heat exposure [7,8,18]. Sex-reversal rates of XX offspring were increased by larval heat exposure (odds ratio ± SE = 3.95 ± 1.99, p = 0.008; Fig. 3c), and regardless of larval thermal treatment the offspring of sex-reversed sires were slightly more likely to undergo sex reversal compared to the offspring of sex-concordant sires (Fig. 3c). 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 (odds ratio ± SE = 1.84 ± 0.95, p = 0.235; Fig. 3b).

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 [20,25,26]. Using this approach, we sampled 137 egg masses across 16 breeding sites over 5 years, and among these we found 6 sibgroups in which the lack of XY offspring could not be attributed to sampling stochasticity. These 6 sibgroups were collected in 3 different years from 4 different populations >6 km from each other (Fig. 1c). This demonstrates that sex-reversed frogs can successfully reproduce not only in the population that we studied in detail here but also at several other sites. Furthermore, 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 contradict this, as the proportion of sex-reversed males (20%) was 5 times higher than the proportion of egg masses sired by sex-reversed males (4%; Fig. 1c).

This unprecedented dataset demonstrates that female-to-male sex-reversed agile frogs are just as fertile as their sex-concordant counterparts, and they are not excluded from reproduction either by male-male competition or by female choice. Although our competition trials had small sample size, they clearly showed that sex-reversed males can compete successfully against similarly sized sex-concordant males. Combining this result with the earlier finding that male body mass did not differ by genotypic sex across multiple populations [26], which is further corroborated by the similar body mass of sex-reversed and sex-concordant males that we found in a large sample of our study population (Fig. 2b inset), we can speculate that sex-reversed males are unlikely to have reduced pairing success in breeding aggregations where males of all sizes occur. Once the male has clasped a gravid female, the chances at successful reproduction may also be similar between sex-reversed and sex-concordant males, based on the results of our fertility trials. However, the "double clutches" that we observed in a few trials suggest that some sex-reversed males might produce less offspring in breeding aggregations due to cryptic or direct female choice. This apparently inferior attractiveness of sex-reversed males may explain our finding across a multitude of breeding populations that sex-reversed males sired a lower proportion of egg masses overall than expected based on their relative frequency among phenotypic males. Although the proportion of egg masses containing XY offspring may be increased by multiple paternity, the latter was too infrequent in other populations [38,46] to account for the discrepancy we observed [47]. Thus, the role of female choice in the reproductive success of sex-reversed males certainly deserves further research, especially since theoretical models predict that female mating preferences can have profound effects on the population-level consequences of sex reversal under environmental change [1]. Interestingly, 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 [20].

Furthermore, our comprehensive data presented here demonstrate that offspring survival is unaffected by sire genotypic sex across multiple stressful periods during early life, including heat waves and Rv infection. This aligns with the finding that thermal tolerance, measured as critical thermal maximum, was not affected by maternal sex reversal in *P. vitticeps* lizards [24]. 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 can move to thermally suitable microhabitats to bolster their immune defence and potentially cure themselves [48]. Thus, our results highlight that the transgenerational effects of sex reversal on the vulnerability to multiple stressors is an important avenue for further experimental research. Strikingly, the taxa that are susceptible to environmental sex reversal include many species that are threatened by the contemporary spread of infectious diseases [29,30], which underscores the urgent need for better understanding the biodiversity consequences of synergistic anthropogenic environmental stressors.

Altogether, the strongest difference we found between sex-concordant and sex-reversed males was that the latter had all-female offspring at the chromosomal level, whereas the former showed the expected 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 [1,2]. 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 [1,2,27]. Our findings support such a diminishing effect, because the offspring of sex-reversed sires were slightly 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. 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.

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Figures

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. **b,** In our experiment, 27 % of females paired with a sex-reversed male spawned their eggs in two consecutive masses (Extended Data Fig. 1). **c,** Across breeding ponds sampled in Hungary, the proportion of sex-reversed males was significantly lower among sires (inferred from the genotypic sex ratios of egg masses spawned in the wild) than among all phenotypic adult males captured in the spawning season (odds ratio ± SE = 0.18 ± 0.11; p = 0.019). Each circle represents a spawning site; the large circle marks the population where the experiment was carried out. 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 [47]).

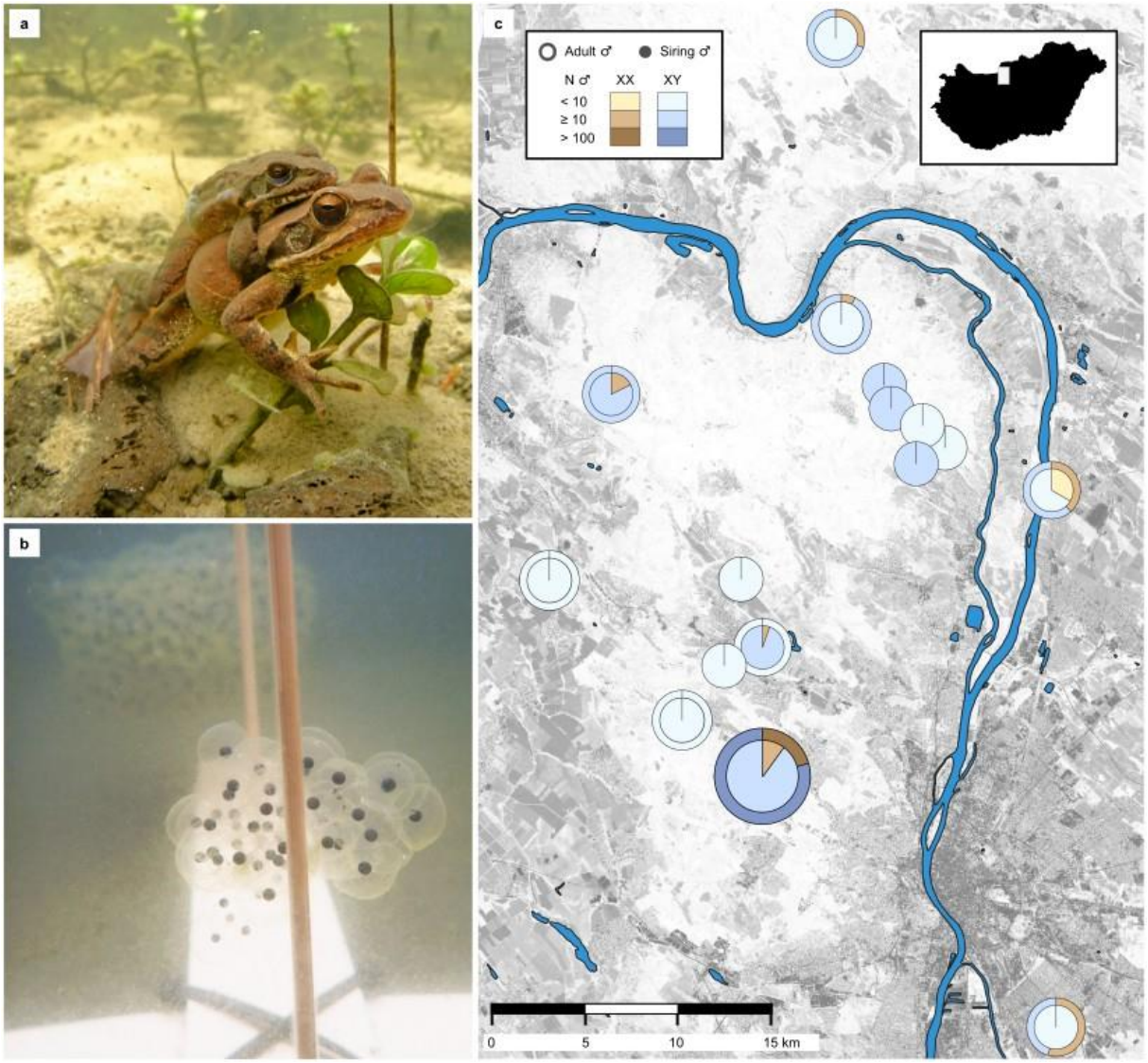


Fig. 2 | Comparison of sex-reversed and sex-concordant males for measures of cryptic female choice (a), male vigor (b), and offspring viability (c-e). For spawning (**a**) and exhaustion (giving up amplexus; **b**), the step lines and the polygons illustrate the cumulative hazard and its 95% confidence interval, respectively; the number at the start of each step line shows the number of participating males. In each violin plot (**a-e**), the black dot and the white box represent the median and interquartile range, whiskers extend to 1.5 × interquartile range, 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 non-significant ($p \geq 0.201$; further details are given in Extended Data Table 1 and the annotated R script [47]).

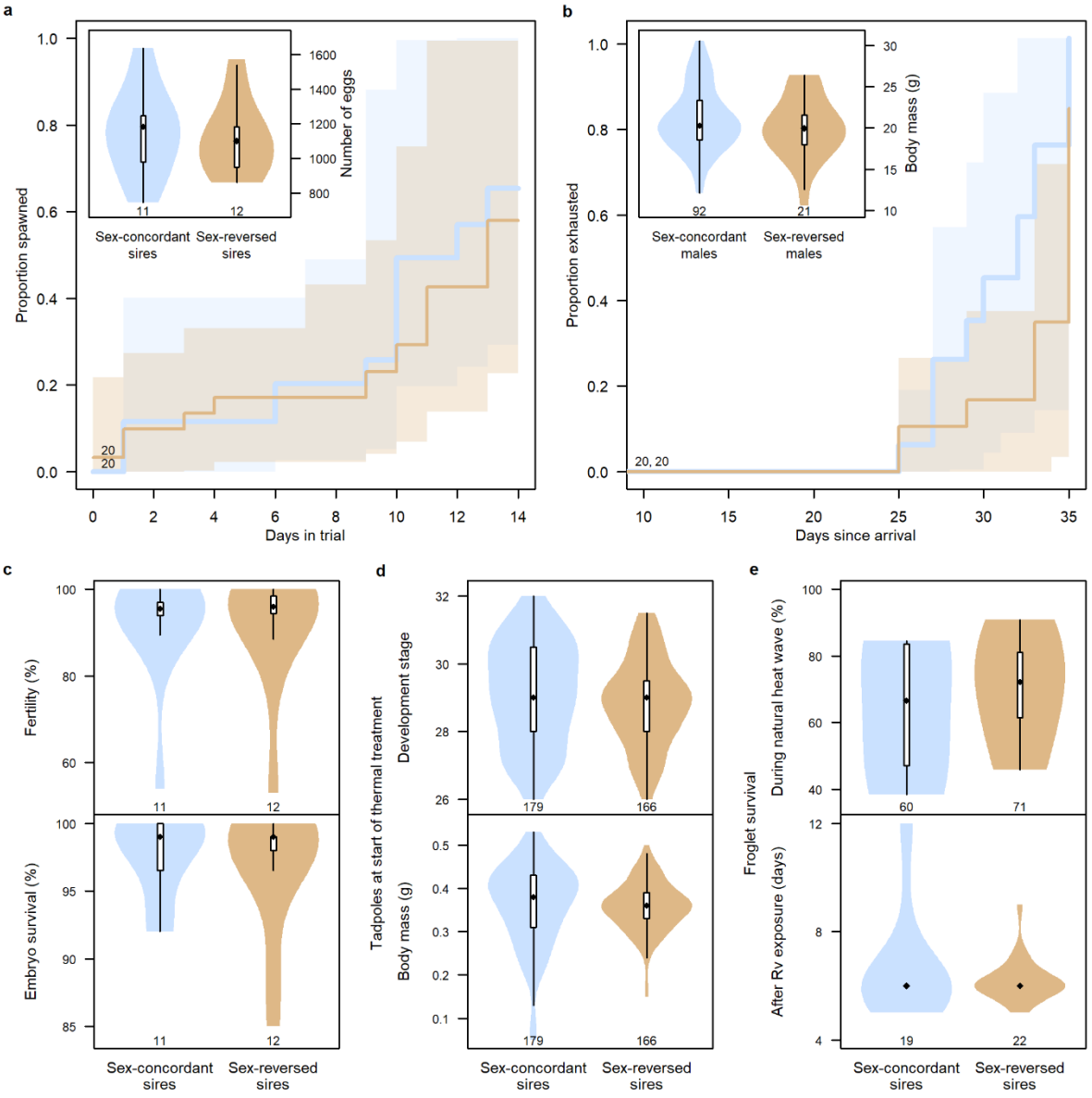
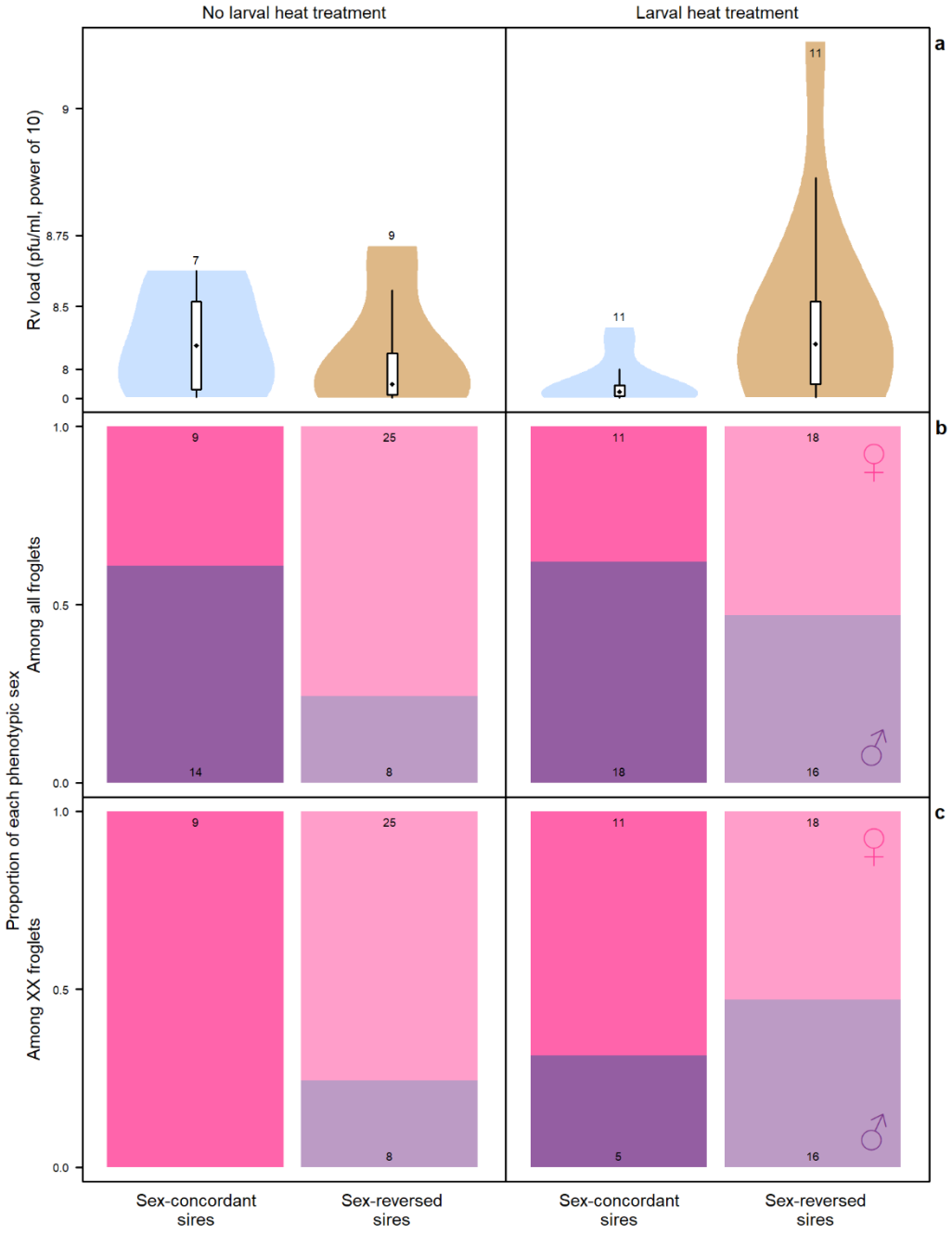


Fig. 3 | Responses by offspring of sex-concordant and sex-reversed males to larval heat exposure and post-metamorphic ranavirus infection. Ranavirus (Rv) load (**a**) in offspring that underwent larval heat treatment was higher for sex-reversed than sex-concordant sires (odds ratio ± SE = 4.38 ± 2.19; p = 0.006), but there was no such difference without larval heat treatment (odds ratio ± SE = 0.57 ± 0.35, p = 0.359). Overall, the progeny of sex-reversed sires had more female-biased phenotypic sex ratio (**b**; odds ratio ± SE = 2.87 ± 1.10; p = 0.006) and a tendency for higher rate of XX-to-male sex reversal (**c**; odds ratio ± SE = 2.89 ± 1.65; p = 0.053) compared to the progeny of sex-concordant sires. Sample sizes are shown above (**a**) or within (**bc**) each plot; see Fig. 2 caption for interpretation of violin plots.



Methods

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 10-11 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-L plastic boxes lined with wet paper towels, and released them next morning into containers (87 × 64 cm) that were placed next to the pond, filled with pond water to ca. 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, 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 [26]. This yielded 21 sex-reversed and 92 sex-concordant males (for 13 males the results were inconclusive [47]). Out of these 113 individuals, we selected 20 duos each consisting of one sex-reversed and one sex-concordant male such that the two males within each duo would be similar in body mass (difference ≤ 1.5 g, median: 0.095, mean ± SD: 0.26 ± 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 Rds1 marker for 4 males whose PCR 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 due to exhaustion.

For the first trial, we used similar containers as 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 ca. 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 ca. 15 cm depth with tap water one week before starting this phase to let the chlorine evaporate. In both kinds of containers (i.e. first and second trial), 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 if 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 two 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), or 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) 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 2-3 weeks there [49], which is somewhat shorter than the maximum duration of the first trial of our experiment (starting on 23 February and ending on 18 March, one 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.

After the first trial, we measured the body mass of the frogs again and released the females into the pond. 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 minutes. 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 greyscale images taken during the night. The second trial ended when eggs had been laid and the male released the female (N=3), or 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 trialwas 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 one 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 5 or 6 small clumps from the clutch (sampling both egg masses if the female deposited her eggs in two masses) totaling

100-132 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 × 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 (Gosner's [50] developmental stage 25), we counted the number of living tadpoles, dead embryos and tadpoles, and 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 if 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 two weeks (14-20 days; mean ± SD: 16.15 ± 2.35). The mesocosms were set up by filling 42×72×30 cm plastic tanks with 65 L tap water, adding 1 L pond water and 40 g 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

514 individuals for further experiments. The remaining tadpoles (up to 8 per mesocosm) were
515 weighed, examined for development stage, and released into the pond. All tadpoles were in
516 similar developmental stages at this time (stages 26-32), corresponding to small limb buds
517 with no toe differentiation yet.
518 The retained tadpoles were exposed to a 6-days heat wave to assess their heat tolerance
519 following the methods described earlier [8,18]. In short, each tadpole was housed individually
520 indoors in a plastic box containing 1.7 L UV-sterilized, fully aerated reconstituted soft water
521 (RSW), and lighting was set to follow the natural photoperiod. In each group of 24 siblings
522 (henceforth sibgroups), 12 tadpoles were kept at control temperature (mean ± SD: 17.31 ±
523 0.12 °C) whereas for 12 tadpoles the water was gradually heated up to treatment temperature
524 (mean ± SD: 27.66 ± 0.41 °C) and kept there for 6 days. Every other day we changed the
525 rearing water and fed the tadpoles with chopped, slightly boiled spinach. At the end of the 6
526 days, we stopped the heating and allowed the water to cool down to control temperature. The
527 next day we moved the tadpoles back into the outdoor mesocosms, each container housing 12
528 siblings that had received the same treatment. Twenty-four days after ending the treatment,
529 when some of the tadpoles reached metamorphic climax (stage 42: appearance of forelimbs),
530 we moved all animals to 8 new mesocosms, such that each mesocosm housed up to 60
531 animals that originated from the same type of sire (sex-reversed or sex-concordant) and
532 underwent the same treatment (control or heated). These mesocosms were 30-cm high, 80-cm
533 diameter containers that we filled to the brim with pond water and added branches and small
534 cork rafts to help the metamorphs leave the water. Each mesocosm was dug into the soil
535 within a 3 × 3 m enclosure made of mosquito net that was 60 cm high above ground and dug
536 20 cm deep into the ground. To keep predators away, we removed large ground beetles by
537 trapping and covered the top of the enclosures with 17-mm chicken wire. The enclosures were
538 shaded by trees and had natural vegetation, but we complemented the naturally available food

539 resources with small crickets (*Acheta domesticus*) once or twice a week. Upon each visit, we
540 checked the enclosures from the outside to search for dead froglets; these checks did not
541 recover corpses while the weather was favorable. However, our region was hit by record-
542 breaking hot and dry weather in July and August; during this time, 43 froglets apparently died
543 from heat stress, as they were found dead in or close to the water in good body condition with
544 no sign of injury. We dissected these corpses to identify phenotypic sex by gonad morphology
545 and stored a tissue sample (hind feet) for genotypic sexing in 96% ethanol.
546 On 27 August, we collected all surviving froglets (N=88) from the enclosures and
547 brought them indoors into a quarantine lab where air temperature was 23.66 ± 0.40 °C and
548 lighting was set to follow the natural photoperiod. We measured their body mass and took
549 buccal swab samples which verified that they were negative to Rv. We housed each froglet
550 individually in a 2-L plastic box covered with a transparent, perforated lid, lined with wet
551 paper towels and a piece of egg carton as shelter. The next day, we exposed each froglet to a
552 5-hour treatment in a 6-cm diameter Petri dish filled with 8 ml RSW, following earlier
553 methods [51]. We exposed half of the froglets recaptured from each enclosure to Rv virions
554 (FV3: Frog Virus 3; ATCC No. VR-567) at a concentration of 3×10^6 plaque forming units
555 (pfu) / ml, while the other half received sham treatment (the nutrient medium supplemented
556 with 2 % fetal bovine serum without the virus). We applied a stratified random sampling
557 design to ensure that the two treatment groups did not differ by body mass, sire genotypic sex,
558 and larval heat treatment (Extended Data Table 1). We checked mortality every day for two
559 weeks. Twice a week, we fed the froglets *ad libitum* with small crickets and sprinkled their
560 boxes with RSW to maintain humidity. When an individual was found dead, we dissected it to
561 identify phenotypic sex by gonad morphology, and we stored a tissue sample (hind feet) for
562 genotypic sexing in 96% ethanol. For measuring Rv infection intensity, we stored two liver
563 lobes in 96% ethanol and measured their mass. The froglets that survived for two weeks were

564 dissected the same way after euthanasia in a shallow water bath of 5 g/L tricaine-
565 methanesulfonate (MS-222) buffered to neutral pH with the same amount of disodium
566 hydrogen phosphate. From the tissue samples of froglets, we identified genotypic sex with the
567 methods used for adult males [26]. From the liver samples and buccal swabs, we measured Rv
568 infection intensity with qPCR [51,52]. Liver samples were negative to Rv for all unexposed
569 froglets, except for one individual with a very low FV3 copy number (1,174 pfu/ml) probably
570 due to contamination during dissection.

571 To assess reproductive potential in a larger number of free-living sex-reversed males, we
572 created two datasets. First, we took our earlier body-mass measurements collected across 11
573 populations (Fig. 1c) from 21 sex-reversed and 89 sex-concordant males [26] and combined it
574 with the data of the 21 sex-reversed and 92 sex-concordant males weighed in the present
575 study upon arrival to the breeding pond. Second, we compiled the data we had collected for
576 previous studies (2018-2023) on the genotypic sex of siblings from 147 egg masses across 16
577 populations [7,8,18,53–55]. In these samples, genotypic sex was identified after several months of
578 raising the animals from eggs in captivity. To test whether these sibgroups originated from
579 sex-reversed sires, we performed a simulation in which we sampled a Bernoulli distribution
580 with a probability of 0.5, representing the proportion of offspring that should inherit the Y
581 chromosome if the sire is sex-concordant. For the sample size of each sibgroup in our dataset,
582 we ran 10,000 iterations and calculated the 99% confidence interval of the proportion of XY
583 offspring. Whenever this confidence interval excluded zero, meaning <1% chance of getting
584 all-XX offspring merely by random sampling from a sex-concordant sire, we concluded that
585 the sibgroup originated from a sex-reversed sire. We used this approach to calculate the
586 proportion of egg masses sired by sex-reversed males for each population, and we compared
587 this to the proportion of sex-reversed males among phenotypic males captured in the 12
588 populations that we sampled as described above for body mass. These two proportions are

589 expected to be similar if sex-reversed and sex-concordant males are equally successful at
590 breeding in nature. Because the simulation showed that the minimally required sample size
591 for identifying a sex-reversed sire with 95% confidence was 6 offspring, we excluded the
592 sibgroups with $N \leq 5$ from further analyses, leaving 137 sibgroups with 16.3 ± 10.4 (mean ±
593 SD; up to 52) siblings in each.

594 Detailed description of our statistical analyses with R [56] scripts and outputs is available
595 along with the datasets [47]. In short, we calculated the latency to spawning as the number of
596 days from the initiation of amplexus to egg laying, and the latency of exhaustion as the
597 number of days from capture to exhaustion (defining exhaustion as the male releasing the
598 female without spawning or not initiating amplexus either with the replacement female in the
599 first trial or with the female in the second trial). We analyzed these data with Cox's
600 proportional hazards models, treating cases where spawning or exhaustion did not happen as
601 censored observations. We estimated fertility rate as the proportion of eggs that developed
602 into embryos, and embryo viability rate as the proportion of embryos that reached stage 25
603 alive. We analyzed these data, as well as the number of egg masses (one or two per female),
604 the proportion of froglets surviving in each enclosure, and the proportion of phenotypically
605 female froglets out of all froglets (i.e. phenotypic sex ratio) and out of XX froglets (i.e. sex-
606 reversal rate) using generalized linear models with binomial error and logit link; we applied
607 Firth's bias reduction method in these models to deal with separation in the number of egg
608 masses and in offspring sex-reversal rates. We used a simple linear model to analyze the
609 number of eggs spawned, and a generalized linear model with negative binomial error and
610 logit link to analyze Rv infection intensity. In all these models, the predictor variable was sire
611 genotypic sex, along with potentially confounding variables such as sire body mass, date, or
612 offspring treatment (for details, see our annotated R code [47]). To compare the body mass of
613 sex-concordant and sex-reversed males captured for this study, we used a generalized least-

squares model that allowed for the two groups to differ in variance. 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 model with binomial error, and we included population as a random factor.

Data availability

The data tables analyzed for this paper are available at Figshare: https://figshare.com/s/54869f7ff693c9de5113 [47].

Code availability

Two annotated R scripts that allows to reproduce the statistical analyses and the figures, respectively, are available at Figshare: https://figshare.com/s/54869f7ff693c9de5113 [47].

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Inclusion and ethics statement

Our study brings together authors from several research groups based in the country where the study was carried out, with roles and responsibilities agreed amongst collaborators ahead of the research. There was no risk to participants. We have included several citations of local and regional research. 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, PE/EA/00270-6/2023).

Acknowledgements

This study was funded by the National Research, Development and Innovation Office of Hungary (NKFIH, grant K-135016 to V.B., PD-134241 to Z.M., PD-142654 to J.U., STARTING-149841 to D.H., and K-147500 to A.H.), the Austrian Science Fund (FWF; grant ESP 239-B to E.N.), and the HUN-REN Hungarian Research Network. The authors were further supported by the strategic research fund of the University of Veterinary Medicine Budapest (Grant No. SRF-001 to E.B. and V.B.), the New National Excellence Program (ÚNKP-23-4 to A.K.), University Excellence Scholarship Program (EKÖP-24 -DOK-06 to

E.B., EKÖP-MATE/2024/25/K to N.U., EKÖP-24-4-II-ELTE-307 to J.U., and EKÖP-24-4-II-ELTE-364 to Z.M) of the Ministry for Culture and Innovation from the source of the National Research, Development and Innovation Fund, and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (to D.H.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript We thank Judit Baumann, Shannon Hogan, Gábor Berkei, István Göcző, Zoltán Örkényi, András Rotter, Mihály B. Rusz, Márk Szederkényi, and Bernadett Zsinka for their help with data collection, and all colleagues and students who helped in the previous experiments from which the clutch sex ratio data were published. The Frog Virus 3 isolate was provided by R.E. Marschang (currently at Laboklin GmbH) to T.P., who was working under her supervision at the Hohenheim University in Stuttgart.

Author contributions

This study was conceptualized by V.B. and E.N., with all authors contributing to the detailed planning of the experiment. All authors participated in data collection; the order of authors from third to ninth reflects their contribution to the work with animals. Molecular work was carried out by E.B. with help from D.H., T.P. and E.N. The ranavirus strain was propagated and titrated by T.P. Statistical analyses and visualization were performed by V.B.; E.N. contributed to data visualization. V.B. wrote the initial draft, and all authors were involved in review and editing.

Competing interests

The authors declare no competing interests.

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690 **Additional information**

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693 **Extended Data Table 1. Sample sizes for froglets sired by sex-concordant or sex-reversed**

694 **males in various treatment groups.**

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	No larval heat treatment		Larval heat treatment		
	Sex-concordant sire	Sex-reversed sire	Sex-concordant sire	Sex-reversed sire	Total
Transferred into froglet enclosures*	108	107	106	106	427
Total died during natural heat wave	**10**	**10**	**9**	**14**	**43**
Analyzed for sex†	8	10	7	14	39
Total survived to Rv experiment	**16**	**26**	**25**	**21**	**88**
Escaped before treatment	0	2	2	0	4
Control (Rv-unexposed)	8	13	11	10	42
Rv-exposed, analyzed for Rv load‡	7	9	11	11	38
Rv-exposed, analyzed for sex§	7	10	11	10	38

*Each of the 4 treatment groups started the thermal treatment with N=120 tadpoles; 5 tadpoles died during or shortly after thermal treatment. The remaining animals were transferred into froglet enclosures around the time of metamorphosis, except for two sibgroups (N=48) that were not transferred due to an accidental error and were released into the pond instead.

†Phenotypic sex is missing for 4 froglets because the corpses recovered from the enclosures during the natural heat wave were too degraded. In one of these individuals, genotypic sexing also failed for the same reason.

‡After Rv exposure, mortality was checked daily and the corpses were sampled for Rv. Data on Rv load are missing for 3 froglets in which the liver was too degraded. Additionally, one froglet died during Rv-exposure so its Rv load was not analysed.

§In 4 Rv-exposed froglets, phenotypic sex was not identifiable because the gonads were too degraded. In one of these individuals, genotypic sexing also failed for the same reason.

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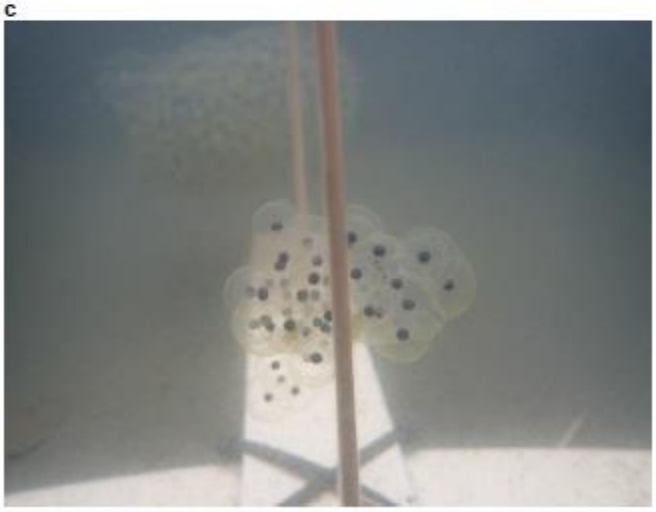
Extended Data Figure 1. Underwater photographs of the "double clutches" laid by three female agile frogs in the fertility trials. Female 29 **(a)** and female 17 **(b)** deposited all their eggs within 24 hours (i.e. between two consecutive daily checks), whereas female 35 **(c)** laid the smaller egg mass (in front) one day later than the larger mass (in the back). Note that in Figure 1b in the main text, the photograph of the "double clutch" of female 35 has been brightened to enhance visibility. The photographs presented here were not manipulated, except for cropping the top and bottom of the uppermost image.

a

b

c



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