



Sex-Biased Mortality and Sex Reversal Shape Wild Frog Sex Ratios

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Population sex ratio is a key demographic factor that influences population dynamics and persistence. Sex ratios can vary across ontogeny from embryogenesis to death and yet the conditions that shape changes in sex ratio across ontogeny are poorly understood. Here, we address this issue in amphibians, a clade for which sex ratios are generally understudied in wild populations. Ontogenetic sex ratio variation in amphibians is additionally complicated by the ability of individual tadpoles to develop a phenotypic (gonadal) sex opposite their genotypic sex. Because of sex reversal, the genotypic and phenotypic sex ratios of entire cohorts and populations may also contrast. Understanding proximate mechanisms underlying phenotypic sex ratio variation in amphibians is important given the role they play in population biology research and as model species in eco-toxicological research addressing toxicant impacts on sex ratios. While researchers have presumed that departures from a 50:50 sex ratio are due to sex reversal, sex-biased mortality is an alternative explanation that deserves consideration. Here, we use a molecular sexing approach to track genotypic sex ratio changes from egg mass to metamorphosis in two independent green frog (*Rana clamitans*) populations by assessing the genotypic sex ratios of multiple developmental stages at each breeding pond. Our findings imply that genotypic sex-biased mortality during tadpole development affects phenotypic sex ratio variation at metamorphosis. We also identified sex reversal in metamorphosing cohorts. However, sex reversal plays a relatively minor and inconsistent role in shaping phenotypic sex ratios across the populations we studied. Although we found that sex-biased mortality influences sex ratios within a population, our study cannot say at this time whether sex-biased mortality is responsible for sex ratio variation across populations. Our results illustrate how multiple processes shape sex ratio variation in wild populations and the value of testing assumptions underlying how we understand sex in wild animal populations.

Keywords: amphibian, endocrine disruption, population dynamics, sex determination, suburban, urban ecology

INTRODUCTION

Sex ratio is a fundamental characteristic of a population capable of affecting its demography and fate (Cotton and Wedekind, 2009; Earl, 2019). In species where gonadal sex is determined by sex chromosomes, Fisher (1930) predicted that sex ratios should be equal at birth, hatching, or metamorphosis. However, observations of sex ratios at early life stages are frequently biased from parity in a variety of species (Hardy, 2002). In mammals and birds, most research on the drivers of early-life sex ratio variation has stressed parental sex allocation – where parents manipulate the sex of their offspring prior to or soon after fertilization by disproportionately allocating resources to a given sex – as a leading mechanism modulating sex ratio in early life stages (Charnov, 1982; West, 2009). However, research in other vertebrates, like many fishes and non-avian reptiles, emphasizes a role for sex determination in shaping offspring sex ratios (Sarre et al., 2004; Ospina-Alvarez and Piferrer, 2008). Sex determination in vertebrates occurs along a spectrum (Sarre et al., 2004). At one end, an organism's phenotypic sex is controlled entirely by genes (genotypic sex determination) and at the opposite end by environmental conditions like temperature (environmental sex determination). In between these extremes there can be roles for both genetic and environmental factors to contribute to sex determination. Such species can sex reverse, where environmental conditions cause individuals to develop their phenotypic gonadal sex opposite from their genotypic sex (Sarre et al., 2004; Quinn et al., 2007; Ospina-Alvarez and Piferrer, 2008; Alho et al., 2010; Holleley et al., 2015, 2016; Lambert et al., 2019; Miko et al., 2021). In mammals and birds, phenotypic sex is genetically determined whereas fishes and reptiles show a diversity of sex-determining modes, including environmental sex reversal (Bachtrog et al., 2014).

Amphibians have been used as model organisms for over a century to study how environmental conditions influence sex ratios in developing animals (King, 1909, 1910; Witschi, 1929; Wallace and Wallace, 2000; Pettersson and Berg, 2007; Lambert, 2015; Lambert et al., 2016a, 2018; Miko et al., 2021). Sex ratio variation in metamorphosing amphibians is largely assumed to be the result of sex reversal and phenotypic sex ratios at metamorphosis are a proxy of sex reversal in laboratory and field studies (Wallace and Wallace, 2000; Pettersson and Berg, 2007; Papoulias et al., 2013; Lambert, 2015; Lambert et al., 2015, 2018). In such studies, female-biased phenotypic sex ratios are assumed to result from male-to-female sex reversal and male-biased phenotypic sex ratios from female-to-male sex reversal. However, this assumption is rarely tested. Evaluating whether sex ratio variation is due to sex reversal has remained elusive, in large part because genotypically sexing amphibians is notoriously challenging (Alho et al., 2010; Lambert et al., 2016b, 2019; Nemeshazi et al., 2020). Recent genomic advances permit the development of sex-linked molecular markers that allow researchers to genotypically sex large numbers of individual amphibians (Lambert et al., 2016b, 2019; Nemeshazi et al., 2020). Sex-linked markers have recently been used to demonstrate that sex reversal is occurring in wild amphibian populations

(Lambert et al., 2016b, 2019; Nemeshazi et al., 2020). Even so, no study has yet to directly evaluate sex reversal as a mechanism underlying phenotypic sex ratio variation.

An alternate, and non-exclusive, hypothesis to sex reversal is that amphibian sex ratio variation is driven by sex-biased mortality (**Figure 1**). Sex-biased mortality prior to birth or hatching has been increasingly observed in wild animal populations, particularly in mammals and birds, but also occasionally in fish (Kruuk et al., 1999; Cichon et al., 2005; Svensson et al., 2007; Orzack et al., 2015; Moran et al., 2016; Kato et al., 2017; Firman, 2019). Multiple factors, including temperature stress and parental condition, have been identified as factors underlying sex-biased mortality, suggesting that sex-biased mortality may not be an adaptive process, as has been suggested for sex allocation, but a maladaptive response to stressful rearing conditions (Kruuk et al., 1999; Goth and Booth, 2005; Eiby et al., 2008; DuRant et al., 2016; Firman, 2019). Thus, sex-biased mortality is important to consider as it can directly shape sex ratios (Szekely et al., 2014).

To our knowledge, sex-biased mortality in pre-metamorphic amphibians remains largely unexplored. As with sex reversal, sex-biased mortality has likely remained understudied due to the technical limitations in genotypically sexing amphibians. Only two studies that we are aware of have considered sex-biased mortality in developing amphibians. One study attempted to infer sex-biased mortality by regressing phenotypic sex ratio variation against mortality rates at metamorphosis (Lambert et al., 2016a). This study concluded that sex ratio variation was due to sex reversal and not sex-biased mortality, however, molecular sex data are necessary to better clarify the processes underlying sex ratio variation. Another laboratory experiment found evidence for sex-biased mortality in tadpoles in response to high temperature even though that was not the intention of the study (Miko et al., 2021). Because sex reversal and sex-biased mortality could co-occur, distinguishing the relative contribution of these processes is crucial as both may produce similar phenotypic sex ratios but different genotypic sex ratios which can have implications for the evolutionary ecology and persistence of populations (Cotton and Wedekind, 2009; Earl, 2019).

In this study, we explore sex-biased mortality from embryogenesis to metamorphosis in two natural populations of the Green Frog (*Rana clamitans*) by sampling the genotypic sex of multiple developmental stages directly from a forested pond and a suburban pond. One of the few studies to assess phenotypic sex ratios in wild metamorphosing amphibians found that metamorphosing *R. clamitans* tadpole phenotypic sex ratios were male-biased in uncontaminated forest ponds and equal or female-biased in suburban ponds that were contaminated by chemicals known to impact sexual differentiation (Lambert et al., 2015). Thus, *R. clamitans* sex ratio variation could suggest female-to-male sex reversal in natural populations and perhaps male-to-female reversal in environments impacted by suburban pollutants. Subsequent work used a novel molecular sexing approach and found that sex reversal was prevalent across 16 populations of free-living *R. clamitans* in both forest and

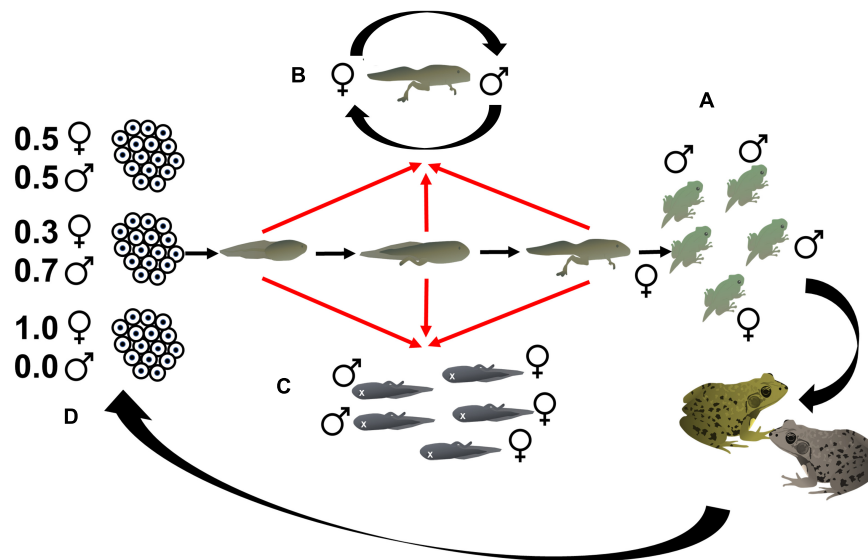


FIGURE 1 | Phenotypic sex ratios (A) in metamorphosing tadpoles may have contributions from the direct effect of sex reversal as tadpoles develop (B), the effects of genotypic sex-biased mortality (C), and indirect effects of sex reversal whereby sex-reversed adults breed and produce clutches with skewed genotypic sex ratios that bias entire cohort sex ratios (D).

suburban ponds and that both female-to-male and male-to-female reversal occur (Lambert et al., 2019). Although this study demonstrated that sex reversal is common in wild *R. clamitans*, it assessed sex reversal in adult frogs. Because adult *R. clamitans* populations represent multiple overlapping offspring cohorts, this study could not determine whether sex reversal contributes to sex ratio variation in metamorphosing *R. clamitans* larvae. Here, our two goals were to (1) document whether sex-biased mortality occurs and alters phenotypic sex ratios and (2) test whether sex reversal is correlated with phenotypic sex ratio variation. We genotype the sex of over 900 *R. clamitans* across ontogeny from embryo to free-swimming tadpole and metamorphosis, illustrating the important contribution of sex-biased mortality, but secondary and inconsistent effects of sex reversal, on phenotypic sex ratios.

MATERIALS AND METHODS

All methods were approved by Yale IACUC protocols 2013-10361 and 2015-10681 and CT DEEP Permit 0116019b.

Ontogenetic Genotypic Sex Ratio Variation

Our goal was to evaluate the existence of sex-biased mortality in wild *R. clamitans* cohorts, inferring bias based on changes in genotypic sex ratios across ontogeny from embryogenesis to metamorphosis (Orzack et al., 2015). We sampled genotypic sex ratios at three developmental points: (1) embryogenesis, when clutches are freshly laid in ponds, (2) the free-swimming tadpole stage, multiple months after hatching and prior to overwintering, and (3) at metamorphosis, a developmental stage analogous to birth in mammals or hatching in birds when aquatic tadpoles

metabolize their tails and develop all four limbs to become more terrestrial. We sampled two ponds – Septic7 and Forest5 – which have been central to our study of sex research in *R. clamitans* and which represent ends of a land use spectrum from undeveloped, forested environments (Forest5) and dense, residential suburbs (Septic7; Lambert et al., 2015).

Clutch Sampling

For clutch sampling, we visited the two ponds between 01 June and 12 July, 2017 every 1–3 days to ensure we sampled all new clutches. We note that this period of time does not typically encompass the entire breeding season of the species but, because of drought conditions, we detected few clutches past 26 June, 2017. We saw reduced breeding after this time because water within the two pond basins dried sufficiently below the emergent vegetation at the ponds' edges that is necessary for *R. clamitans* to deposit clutches. While this survey period is not as long as it would be in most years, it is comprehensive of the breeding season for this year and allows us to begin addressing the degree of sex ratio variation in *R. clamitans* clutches and the genotypic sex ratio for the year's cohorts. From each fresh clutch observed, we collected a subsample of ca. 150 embryos prior to neurulation (Gosner 14 and below), attempting to minimize any environmental effects on embryo survival. This species produces clutches with well over 1,000 embryos and so our samples represent at most 10% of a clutch. Because *R. clamitans* lays a single-layer film of embryos on the water's surface, sampling randomly throughout the egg mass is impossible without destroying the entire mass. As such, we haphazardly sampled around each clutch's periphery in a way that minimized damage to collected embryos or remaining embryos and in a way that did not detach the egg mass from emergent vegetation.

We maintained each clutch in a separate aquarium with reconstituted distilled water at 18°C until embryos freed themselves from jelly which is particularly important for *R. clamitans* because the jelly coat is too sticky to extract individual embryos without destroying them and collecting embryos singly (Sakisaka et al., 2000; Alho et al., 2010). The assumption in our approach is that the lab hatching accurately reflects the genotypic sex ratios of the clutches when collected in the field. We haphazardly collected 28–30 recently hatched embryos from each clutch, shipped tissue samples to Diversity Arrays Technology (Bruce, ACT, Australia), and genotypically sexed all individuals using DArTmp methods including two rounds of polymerase chain reaction and sequencing on an Illumina HiSeq 2500 using a sex-specific molecular marker for *R. clamitans* as described previously (Lambert et al., 2016b, 2019). For the sex-specific marker, we used the locus RaclCT001 which has previously been shown to be perfectly linked to genotypic sex, can accurately diagnose sex reversal on its own in the absence of other sex markers, and for which DArTmp methods consistently provide substantial reads to confidently call sex genotypes (Lambert et al., 2016b, 2019). Importantly, these markers have all been shown to be effective at genotypically sexing *R. clamitans* in all the focal ponds studied here (Lambert et al., 2019). Clutch sex ratio data are provided as **Supplementary Data Sheet 1**.

Larval and Metamorph Sampling

Larval *R. clamitans* have a relatively long larval development for amphibians where larvae typically hatch in late summer, overwinter as larvae in their natal pond, and metamorphose the following summer roughly 1 year after fertilization. From the two cohorts for which we had genotyped sex ratios in wild clutches in June and July 2017, we followed genotypic sex ratios during larval development. Specifically, we collected larvae in October 2017 prior to overwintering and we collected metamorphs in June 2018. Thus, we sampled tadpoles and metamorphs ~95 and 337 days post-hatching, respectively, after we collected the final clutch in July 2017. We sampled larvae and metamorphs by comprehensively dipnetting the entirety of each waterbody, which should provide a relatively unbiased sample of each population. The phenotypic (i.e., gonadal) sex of tadpoles collected in October 2017 were not developmentally differentiated enough to be identifiable as ovaries or testes via dissection (Foote and Witschi, 1939; Lambert et al., 2016b) and so were only genotypically sexed. However, metamorphosing *R. clamitans* have well-differentiated gonads and so we assessed metamorph phenotypic sex by dissection as described previously (Lambert et al., 2015, 2016b). We also genotypically sexed all dissected metamorphs as described above. Larval and metamorph data are provided as **Supplementary Data Sheets 2, 3**.

Analysis

To evaluate whether genotypic sex ratios varied across ontogeny, we took an information theoretic approach to model our data. Specifically, we used binomial generalized linear models (GLMs) to model the frequency of each genotypic sex as a function of Pond (Forest5 or Septic7), developmental Stage (embryo, tadpole,

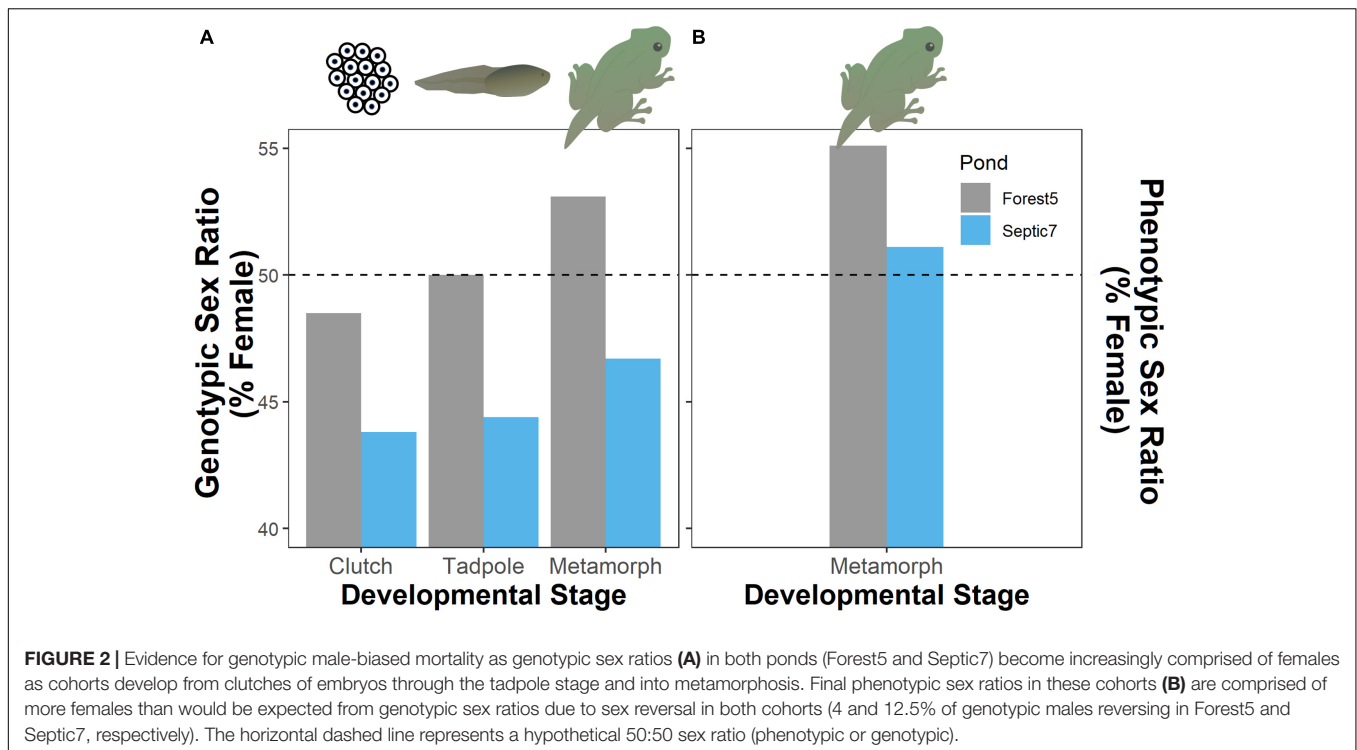
and metamorph), and the effects of Pond and Stage together. We used the *aictab* function in the R (v 3.4.0) package “AICcmodavg” to compare AICc values and model weights for a null (intercept-only) model, each univariate model, and the additive model. We considered models to provide important explanatory power if they were within $\Delta AICc \leq 2.0$ of the top-ranked model (i.e., model with lowest AICc) and carried ≥ 0.10 of model weight. If the null model was the top-ranked model, a candidate model was not considered strong evidence but its trend was considered of interest if $\Delta AICc \geq 2.0$ of the null model and carried ≥ 0.10 of model weight. We constructed models using individuals as the unit of measurement and, because the number of embryo samples were much larger than the tadpole or metamorph samples, we excluded the tadpole samples to minimize the number of contrasts our models had to make between developmental stages. As such, this approach analyzes changes in genotypic sex frequencies from embryo to metamorphosis but does not incorporate an intermediate developmental stage, although these data are presented (**Figure 2**). We checked for the top models' fits by plotting residuals against predicted model estimates because small sample sizes in terms of the number of populations studied did not allow for more integrative model diagnostics. The associated R script and data csv are in **Supplementary Data Sheets 4, 5**.

We also explored whether individual clutch sex ratios in *R. clamitans* had the potential to differ from parity. Because sex ratios can require large sample sizes to detect deviations from parity using traditional frequentist statistical binomial approaches (e.g., Chi-squared analysis), we analyzed individual clutch sex ratio data within a probabilistic Bayesian framework using the maximum *a posteriori* (*map*) function in the R package “rethinking” following McElreath (2016). We only performed this analysis on clutch sex ratios as we did not analyze deviations from parity in sex ratios from other life stages. To test whether clutch genotypic sex ratios were skewed from parity, we used the *map* function and a binomial likelihood to assess whether the proportion of females in a sample differed from a flat prior (i.e., differed from an equal sex ratio). For each clutch we assessed deviation from parity using, for example, a script like: `precis(map(alist(f~ dbinom(30, p), p~ dunif(0,1)), data = list(f = 11)))`. In this example, the script tests for a sex ratio bias in a sample of 30 individuals where 11 are females.

Phenotypic Sex Ratio Relationships With Sex Reversal

Sex Ratios and Sex Reversal

Next, our goal was to understand the relationship – if any – between phenotypic sex ratio variation at metamorphosis and the frequency of sex reversal in either direction (female-to-male or male-to-female reversal). If sex reversal is the primary mechanism underlying phenotypic sex ratio variation, then male-biased phenotypic sex ratios would be correlated with higher frequencies of female-to-male sex reversal, and *vice versa* for female-biased phenotypic sex ratios. To do so, we leveraged specimens collected the prior year across six populations. In 2016, we collected metamorphosing *R. clamitans* tadpoles from six



ponds along a forest-suburban environmental gradient, including the two ponds (Forest5 and Septic7) sampled for the sex-biased mortality study. Because larval *R. clamitans* have a relatively long larval development for amphibians (~1 year), *R. clamitans* larvae are sensitive to drought as ponds can dry in late summer or fall, resulting in complete mortality of entire larval cohorts prior to metamorphosis the following year. Because of the drought conditions, metamorphs were only present in a subset of ponds ($n = 6$; AV08, Septic3, Septic7, Septic10 Sewer3, Forest5) used in prior studies and which maintained water over winter (Lambert et al., 2015, 2019). Even so, the ponds sampled here cover the extent of a forest-suburban environmental gradient used in prior studies of sex ratios and sex reversal (Lambert et al., 2015, 2019). We surveyed ponds at least twice per week, collecting metamorphosing *R. clamitans* larvae from these six ponds as they emerged in summer 2016 and assessed gonadal sex via dissection to estimate phenotypic sex ratios. To infer sex reversal, we genotyped a random subset of metamorphs' sex (minimum of 55% of individuals in a cohort, but typically 70% or more) and identified individuals where genotypic sex differed from phenotypic sex.

Environmental Correlates

We also used these data to explore whether sex ratios and sex reversal were associated with differences in environmental conditions across populations. Although in our prior work we found that sex reversal occurred in both the female-to-male and male-to-female directions and was prevalent across 16 *R. clamitans* populations, including four forested and 12 suburban populations, we were unable to confidently assess environmental relationships with sex reversal frequencies

(Lambert et al., 2019). This is because our prior study assessed sex reversal in adult *R. clamitans* and relating a single year of environmental data to sex reversal frequencies in adults is not possible because this species survives up to 7 years and adult populations comprise multiple overlapping cohorts (Shirose and Brooks, 1995). By assessing sex ratios and sex reversal at metamorphosis here, we can more confidently explore environmental relationships with sex ratios and sex reversal. We collected water condition data [specific conductance (i.e., conductivity), pH, dissolved oxygen, and temperature] at each survey using an Oakton PCSTestr 35 Multiparameter probe (conductivity, pH, and temperature) and a YSI ProODO Optical Meter for dissolved oxygen as described previously (Lambert et al., 2019). We used the mean measurement across surveys for each environmental condition in our statistical analyses. Using GIS and custom high-resolution suburban land use data, we also calculated the percent of land cover surrounding each pond that was either forest or suburban development (Lambert et al., 2019). Our prior work has found that, although environmental conditions certainly fluctuate throughout the year within a pond, relative differences among ponds are generally constant (Lambert et al., 2019). This is important here because the developmental period where *R. clamitans* larval sex determination is sensitive to environmental conditions is unclear. As such, our environmental correlates can help identify patterns of relative environmental conditions associated with sex.

Analysis

We used a similar modeling approach as described in the sex-biased mortality portion of our study. To analyze whether sex ratios varied as a function of sex reversal frequency, we

again used binomial GLMs with phenotypic sex frequency as a binary response variable and assessed the independent and additive influence of female-to-male and male-to-female sex reversal frequencies on phenotypic sex ratios. We similarly modeled the relationship between environmental conditions and phenotypic sex ratios using the same approach except that we used each pond as the unit of measurement and express sex ratios as the proportion of females (individuals with phenotypic ovaries) and sex reversal rates as proportion of genotypic females with testes (female-to-male reversal) or genotypic males with ovaries (male-to-female reversal). Finally, we modeled environmental relationships and either direction of sex reversal separately. For female-to-male sex reversal, our response variable was a binary probability of a genotypic female having either testes or ovaries (i.e., a phenotypic male or female, respectively). We used the same modeling approach for male-to-female sex reversal but using genotypic males. Because the number of sampled populations ($n = 6$) was relatively low due to drought conditions, we only modeled the additive effects of at most two explanatory variables in a single model to avoid overfitting. The associated R script and data csv are in **Supplementary Data Sheet 6**.

RESULTS

Ontogenetic Genotypic Sex Ratio Variation

In total, we genotyped 598 embryos from seven and 13 clutches, respectively, from the two ponds Septic7 ($n = 208$ embryos total) and Forest5 ($n = 390$ embryos total). We sampled 36 tadpoles from each pond and genotyped the sex of all tadpoles. For metamorphs, we sampled 49 frogs from Forest5 and 45 from Septic7, genotyping all metamorphs. By tracking genotypic sex ratios from embryogenesis through metamorphosis in these cohorts, we found that genotypic sex-biased mortality – specifically male-biased mortality – contributed to phenotypic sex ratios in metamorphosing frog cohorts. In both ponds, genotypic sex ratios become more female-biased as larvae developed from embryos to tadpoles through metamorphosis (**Figure 2**). Our analysis found evidence for sex-biased mortality (**Table 1**). In this analysis, an intercept-only model was ranked highest but a model including Stage received sufficient support to infer consistent male-biased mortality between both ponds. Additionally, male-to-female sex reversal caused the final phenotypic sex ratios to become further female-biased relative to the initial genotypic sex ratios of these cohorts' clutches (**Figure 2**).

Interestingly, individual clutch genotypic sex ratios varied from 36.7 to 63.3% female. Our analyses support male-biased genotypic sex ratios for four clutches, two from Septic7 (S7_1 and S7_6) and two from Forest5 (F5_1 and F5_2; **Figure 3**). The cohort genotypic sex ratio estimated by summing across clutches for Septic7 also had a significant male bias (**Figure 3**). There was also support for two clutches from Forest5 (F5_4 and F5_13) exhibiting a genotypic female-bias (**Figure 3**).

Phenotypic Sex Ratio Relationships With the Environment and Sex Reversal

From across the six ponds, we phenotypically sexed 221 ($n = 29–56$) metamorphosing *R. clamitans*. Phenotypic sex ratios in metamorphs from the six focal ponds in 2016 ranged from 39–52% female. We did not identify any environmental correlates of phenotypic sex ratios. Of our models evaluating possible environmental correlations with phenotypic sex ratios, no model including environmental variables improved model fit over a null, intercept-only model all models ($\Delta\text{AICc} > 4.3$ over null model, $\text{AICc Weight} \leq 0.08$; **Table 2**).

We genotyped the sex of a random subset of these metamorphosing *R. clamitans* larvae from each pond ($n = 164$ total; $n = 18–31$ per pond) and inferred sex reversal by identifying differences between each metamorph's genotypic and gonadal sex (i.e., ovaries vs. testes). Consistent with prior work in adult *R. clamitans* (Lambert et al., 2019), we found both female-to-male and male-to-female sex reversal directions, each occurring across roughly half of the study populations. Neither direction of sex reversal provided a better fit to the phenotypic sex ratio data than an intercept-only model (**Table 2**), suggesting sex reversal is not tightly or consistently associated with phenotypic sex ratios.

Environmental Predictors of Sex Reversal

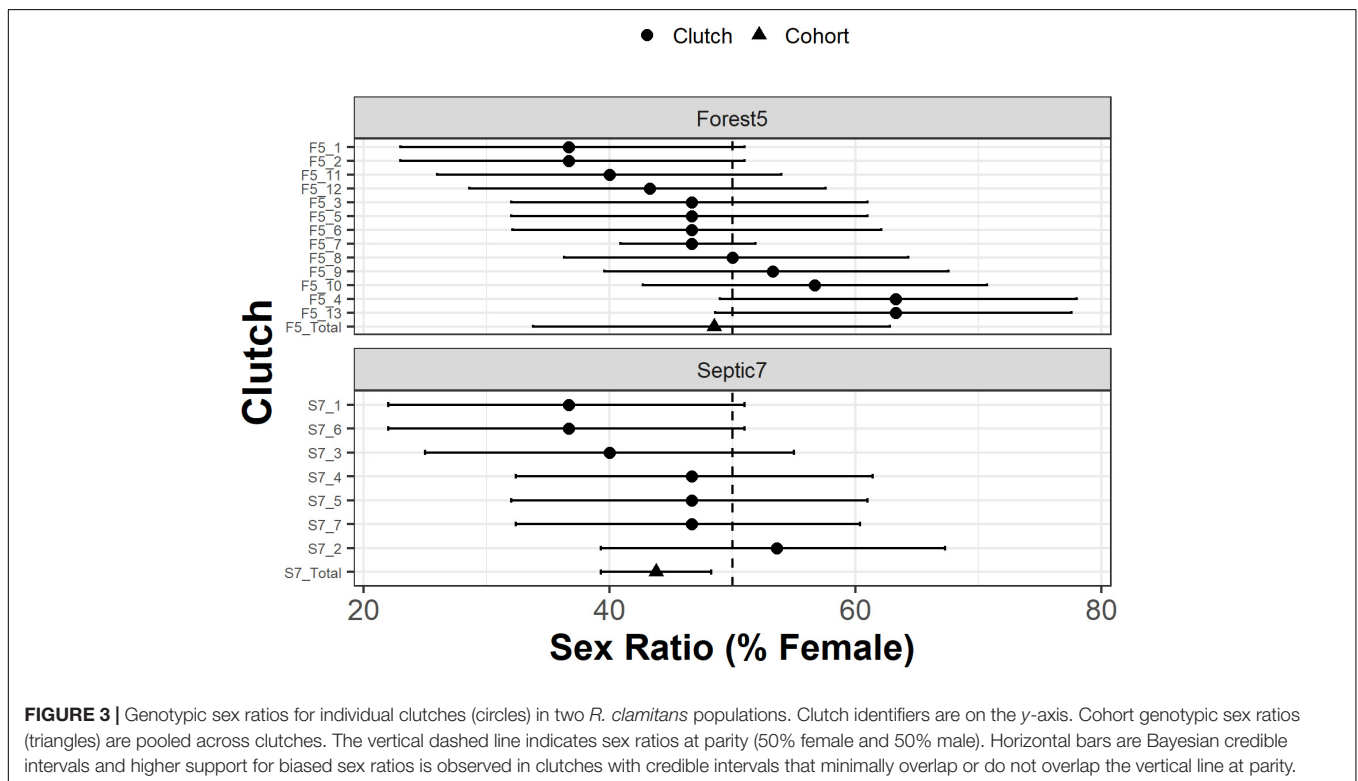
Female-to-male sex reversal was best predicted by dissolved oxygen concentrations in ponds with a univariate model carrying $> 50\%$ of model weight (**Figure 4** and **Table 3**). Models containing pH, dissolved oxygen + temperature, and temperature alone were also relevant candidate models for female-to-male sex reversal in metamorphosing *R. clamitans* (**Figure 4** and **Table 3**). In sum, rates of female-to-male sex reversal were highest in metamorphosing tadpoles developing in ponds with low dissolved oxygen, more acidic pH, and cooler pond temperatures.

No environmental variables explained male-to-female sex reversal (i.e., an intercept-only model was the best supported model; **Table 3**). However, univariate models containing suburban land use and mean pond water temperatures carried $> 24\%$ of AICc model weight each and were within 2.0 ΔAICc from the null model (**Table 3**). These results provide limited evidence that rates of male-to-female sex reversal were highest in ponds impacted by surrounding suburban land use and ponds with warmer average water temperatures.

We note that, for the two focal populations we studied for the sex-biased mortality part of the study in 2018 and in the sex reversal part of our study, the extent of sex reversal in metamorphosing cohorts varies between populations and among years within a population (**Figure 5**). Additionally, our focal environmental variables (dissolved oxygen, temperature, and pH) and suburban land use were largely uncorrelated in this study (linear regression, all $p \geq 0.14$), except for a correlation between pond pH and dissolved oxygen (linear regression, $p = 0.009$, $R^2 = 0.81$).

TABLE 1 | Analysis of models of genotypic sex ratio across developmental stages (a proxy of sex-biased mortality) and between ponds using individual sample as the unit of measurement.

	Variables	AICc	Delta_AICc	AICcWt	Cum.Wt
Genotypic sex ratio: Both ponds	Intercept only	959.23	0.00	0.38	0.38
	Pond	959.82	0.58	0.28	0.66
	Stage	960.92	1.68	0.16	0.82
	Stage + Pond	961.36	2.13	0.13	0.95
	Stage × Pond	963.36	4.13	0.05	1.00
Genotypic sex ratios: Forest5	Intercept only	610.41	0.00	0.70	0.70
	Stage	612.06	1.65	0.30	1.00
Genotypic sex ratios: Septic7	Intercept only	349.42	0.00	0.72	0.72
	Stage	351.32	1.90	0.28	1.00



DISCUSSION

The pattern of genotypic sex ratio in multiple populations from the embryo stage to metamorphosis is consistent with an important role for sex-biased mortality in amphibians. Although there is evidence of sex-biased mortality in juvenile mammals, birds, and even some fish (Kruuk et al., 1999; Cichon et al., 2005; Svensson et al., 2007; Orzack et al., 2015; Moran et al., 2016; Kato et al., 2017; Firman, 2019), to our knowledge, this is the first record of sex-biased mortality in larval amphibians in the wild. Importantly, we find the same pattern of male-biased mortality across two independent frog populations which inhabit contrasting environments, suggesting sex-biased mortality may be a relatively common phenomenon. Although we identified both directions of sex reversal, we found little evidence that sex reversal frequencies are consistently

associated with phenotypic sex ratios. Even though sex reversal may not be the dominant mechanism undergirding phenotypic sex ratio variation, sex reversal still contributes to phenotypic sex ratios. In the populations we studied, male-biased mortality produced proportionally more genotypic females over larval development and additional sex reversal led to proportionally more phenotypic females than expected from genotypic sex ratios alone. The degree of sex-biased mortality we detected was modest (3–5% percentage change) but the degree of sex ratio change due to sex-biased mortality is sufficient to have important demographic consequences (Wedekind, 2017). To be clear, we identified sex-biased mortality as a mechanism that shapes sex ratios of individual wild amphibian populations, but our study was not designed to document whether sex-biased mortality shapes sex ratio variation across populations. Additionally, we note that our limited sample size of six populations may have had

TABLE 2 | Models of phenotypic sex ratios as a function of environment and sex reversal.

	Variables	AICc	Delta_AICc	AICcWt	Cum.Wt
Environmental predictors	Intercept only	26.92	0.00	0.71	0.71
	pH	31.31	4.39	0.08	0.79
	Temp	31.38	4.47	0.08	0.86
	DO	31.48	4.56	0.07	0.94
	Suburban	31.88	4.97	0.06	1.00
	Suburban + Temp	40.54	13.63	0.00	1.00
	pH + Temp	41.03	14.11	0.00	1.00
	Temp + DO	41.09	14.17	0.00	1.00
	pH + DO	41.29	14.37	0.00	1.00
	Suburban + pH	41.31	14.39	0.00	1.00
	Suburban + DO	41.48	14.56	0.00	1.00
Sex reversal predictors	Intercept only	26.92	0.00	0.83	0.83
	Female-to-Male sex reversal	31.27	4.35	0.09	0.93
	Male-to-Female sex reversal	31.81	4.90	0.07	1.00
	Female-to-Male + Male-to-Female sex reversal	41.22	14.31	0.00	1.00

limited power to detect a relationship between sex reversal and phenotypic sex ratios, although the lack of a detectable pattern with these six population is notable. Sex-biased mortality, to our knowledge, is a previously undocumented process in larval amphibians and acts in concert with other processes like sex reversal to shape the phenotypic sex ratios of entire cohorts.

The causes for sex-biased mortality in this system remain to be studied, but both chromosomal and ecological processes may contribute. Heterogametic sex chromosomes (Y-chromosomes in species with XX-XY sex chromosome and W-chromosome in species with ZZ-ZW sex chromosomes) are often degenerate and accumulate deleterious alleles (Charlesworth and Charlesworth, 1997; Gordo and Charlesworth, 2001; Bachtrog, 2008; Miura et al., 2012; but see Kratochvil et al., 2021). As such, these degraded sex chromosomes can reduce survival and longevity in a diversity of animals (Xirocostas et al., 2020). Given *R. clamitans* has an XX-XY sex chromosome system (Lambert et al., 2016b), *R. clamitans* Y-chromosomes may be degraded (although there is no evidence for sex chromosome heteromorphy) which could explain higher rates of genotypic male mortality early in life. The absence of YY-individuals in our study may provide additional support for this hypothesis if YY-embryos are frequently unfit, as is often expected (Bull, 1983; Charlesworth, 1996). However, embryos with two Y-chromosomes may also never exist in wild populations if ecological factors limit reproduction by male-to-female reversed frogs.

Ecological differences between genotypic female and male larvae may also contribute to sex-biased mortality. For instance, pilot laboratory data suggest that male wood frog (*Rana sylvatica*) tadpoles exhibit higher activity patterns than female tadpoles (Skelly et al., unpubl.). Higher activity in tadpoles is associated with higher mortality due to predation (Skelly, 1994). If *R. clamitans* larvae similarly display sex-specific behaviors, then there is the possibility for sex-biased predation that could contribute to the sex-biased mortality we observed. Further, sex-specific behaviors may also influence sex-specific sampling efficacy. For instance, if sexes differ in microhabitat preferences,

different types of sampling approaches may be biased toward one sex or another. Although identifying this issue is beyond the scope of our study, we sampled through the entire water body of each pond, including the benthos and surface and so presumably this bias should not influence our results. In mammals and birds, evidence also suggests that stress or poor health in parents can lead to sex-biased mortality in embryos and offspring (Kruuk et al., 1999; Muller et al., 2005; Perez et al., 2006; Firman, 2019). It is possible that reduced body condition in breeding *R. clamitans* similarly contributes to sex-biased mortality of embryos and tadpoles. Additionally, sex-biased mortality may be a result of female and male differences in tolerance of various environmental conditions like temperature, as has been seen in birds (Goth and Booth, 2005; DuRant et al., 2016; Eiby et al., 2008). Support for these and other hypotheses require further research.

Our analyses provide some evidence for environmental conditions associated with sex reversal. Specifically, our data suggest that natural water chemistry (dissolved oxygen, pH, and temperature) are related to genotypic female *R. clamitans* developing as phenotypic males. Experimental evidence in fishes shows that sex reversal can result from variation in dissolved oxygen and pH, and so these conditions may be relevant to amphibian sexual development (Romer and Beisenherz, 1996; Oldfield, 2005; Shang et al., 2006). We also have correlative evidence of temperature-mediated sex reversal where cooler temperatures contribute to higher rates of female-to-male sex reversal. Intriguingly, the opposite trend occurred for male-to-female sex reversal where warmer temperatures contribute to more male-to-female reversal, although the strength of evidence was weak. Temperature-mediated sex reversal in amphibians has been explored in laboratory experiments (Witschi, 1929; Wallace and Wallace, 2000; Lambert et al., 2018) but, to our knowledge, this is the first evidence from wild populations. Sex reversal in amphibians has, in recent decades, been assumed to be strictly caused by environmental pollutants or to unnatural conditions imposed by laboratory experiments (Hayes, 1998;

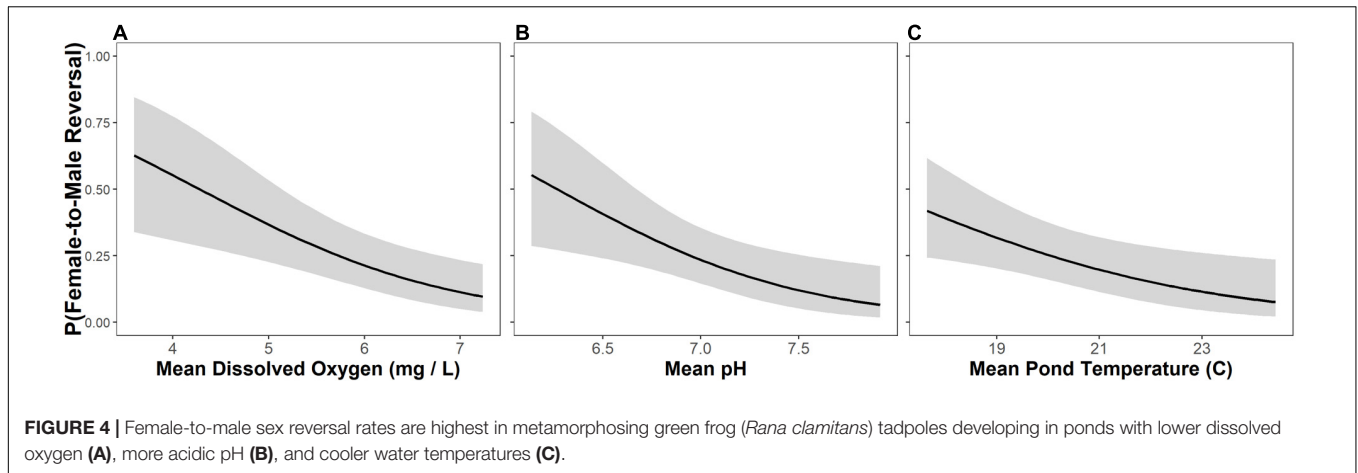


TABLE 3 | Model ranking for environmental predictors of sex reversal.

	Variables	AICc	Delta_AICc	AICcWt	Cum.Wt
Female-to-Male sex reversal	DO	30.46	0.00	0.51	0.51
	pH	32.95	2.49	0.15	0.66
	DO + Temp	33.36	2.89	0.12	0.78
	Temp	33.81	3.35	0.10	0.88
	Intercept only	35.56	5.10	0.04	0.92
	pH + Temp	35.71	5.24	0.04	0.95
	Suburban + DO	36.60	6.14	0.02	0.98
	Suburban	38.59	8.13	0.01	0.99
	Suburban + pH	38.94	8.48	0.01	0.99
	DO + pH	39.70	9.23	0.01	1.00
	Suburban + Temp	41.56	11.09	0.00	1.00
Male-to-Female sex reversal	Intercept only	24.87	0.00	0.32	0.32
	Suburban	25.13	0.26	0.28	0.61
	Temp	25.41	0.54	0.25	0.86
	DO	28.36	3.49	0.06	0.91
	pH	29.09	4.22	0.04	0.95
	Suburban + pH	30.97	6.10	0.02	0.97
	Suburban + Temp	31.49	6.62	0.01	0.98
	pH + Temp	32.02	7.15	0.01	0.99
	DO + Temp	32.26	7.39	0.01	1.00
	Suburban + DO	35.06	10.19	0.00	1.00
	DO + pH	37.87	13.00	0.00	1.00

Eggert, 2004; Nakamura, 2013; Orton and Tyler, 2015). This contrasts with work over a century ago that assumed sex reversal was largely a natural process (King, 1909, 1910; Witschi, 1929). Our findings here and previously in both metamorphosing and adult *R. clamitans* – in addition to recent work on European Agile Frogs (*Rana dalmatina*) – suggest that sex reversal can be natural and not necessarily a response to abnormal conditions because sex reversal has been observed under natural conditions in the wild for both species (Lambert et al., 2019; Nemeshazi et al., 2020). The conditions (dissolved oxygen, pH, and temperature) correlated with sex reversal vary naturally but can also be influenced by human land use (Holgerson et al., 2018; Lambert et al., 2019). However, in our study, these variables were uncorrelated with suburban land use (all $p \geq 0.14$), suggesting

that female-to-male reversal is largely natural. Although, our analysis did provide limited support that suburban land use increased the rate of male-to-female sex reversal. These patterns should encourage further work on sex reversal and the relative effect of natural and anthropogenic causes in wild amphibians.

Temporal variation may complicate identifying relationships between the environment, sex ratios, and sex reversal. For instance, in our two focal populations (Forest5 and Septic7), the presence, direction, and frequency of sex reversal varied not only between the two populations but also between years (Figure 5). This resulted in among-year differences in phenotypic and genotypic sex ratios (Figure 6). Among-year variation may explain why our results seemingly contrast with previous research. In prior work in this system, metamorphs

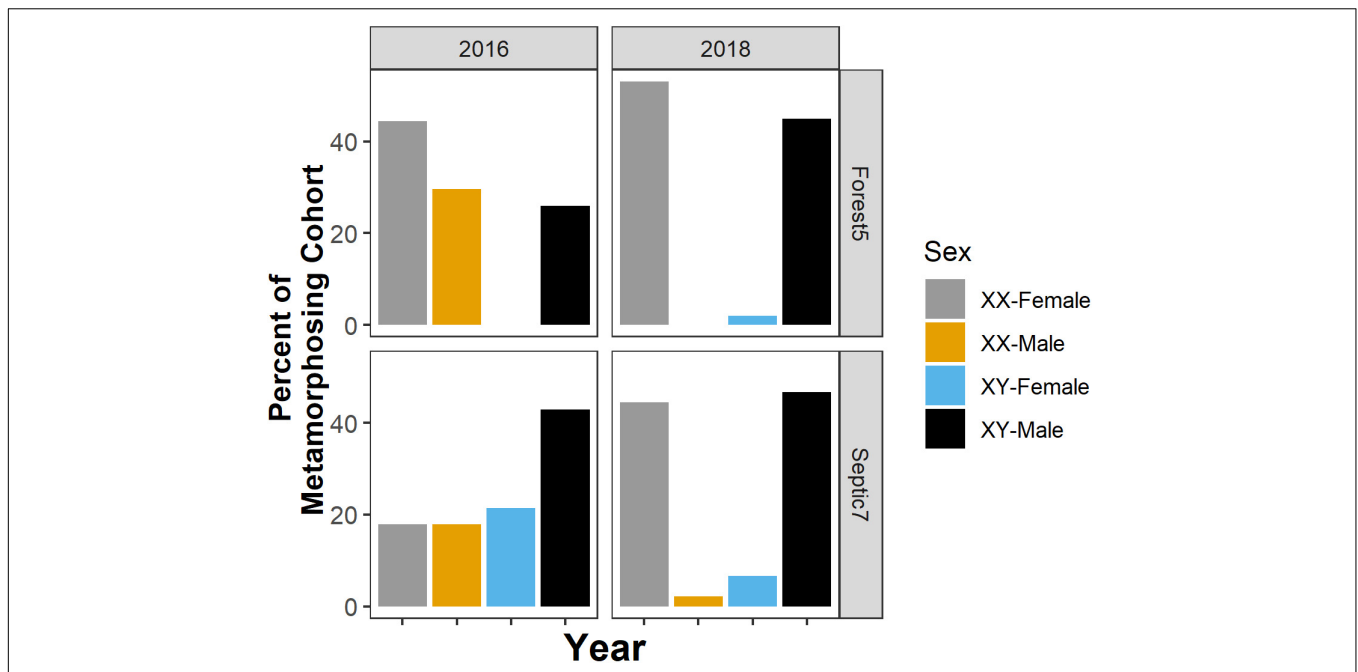


FIGURE 5 | The presence, direction, and frequency of sex reversal in metamorphosing cohorts varies between populations and among years within a population. Sexual genotype-phenotype data from two focal ponds – Forest5 and Septic7 – in the years 2016 and 2018. Orange and blue bars represent frequencies of sex-reversed metamorphs and gray and black bars represent non-reversed frogs.

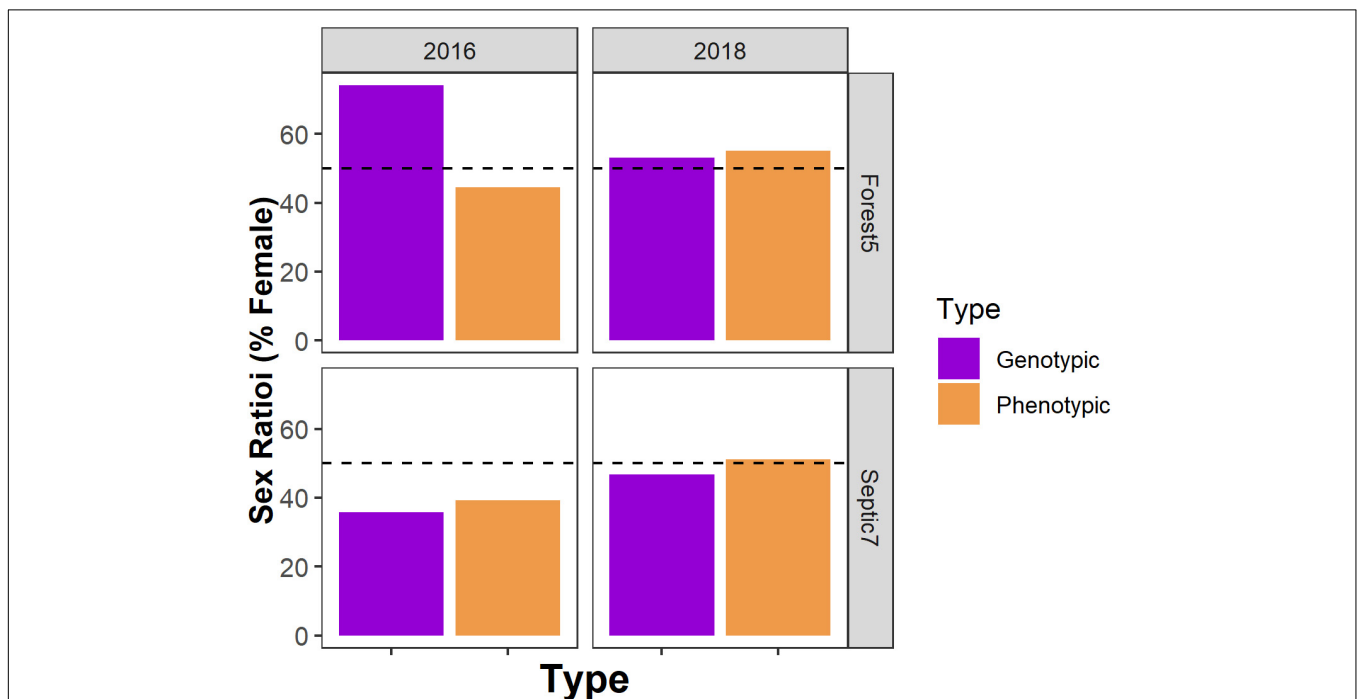


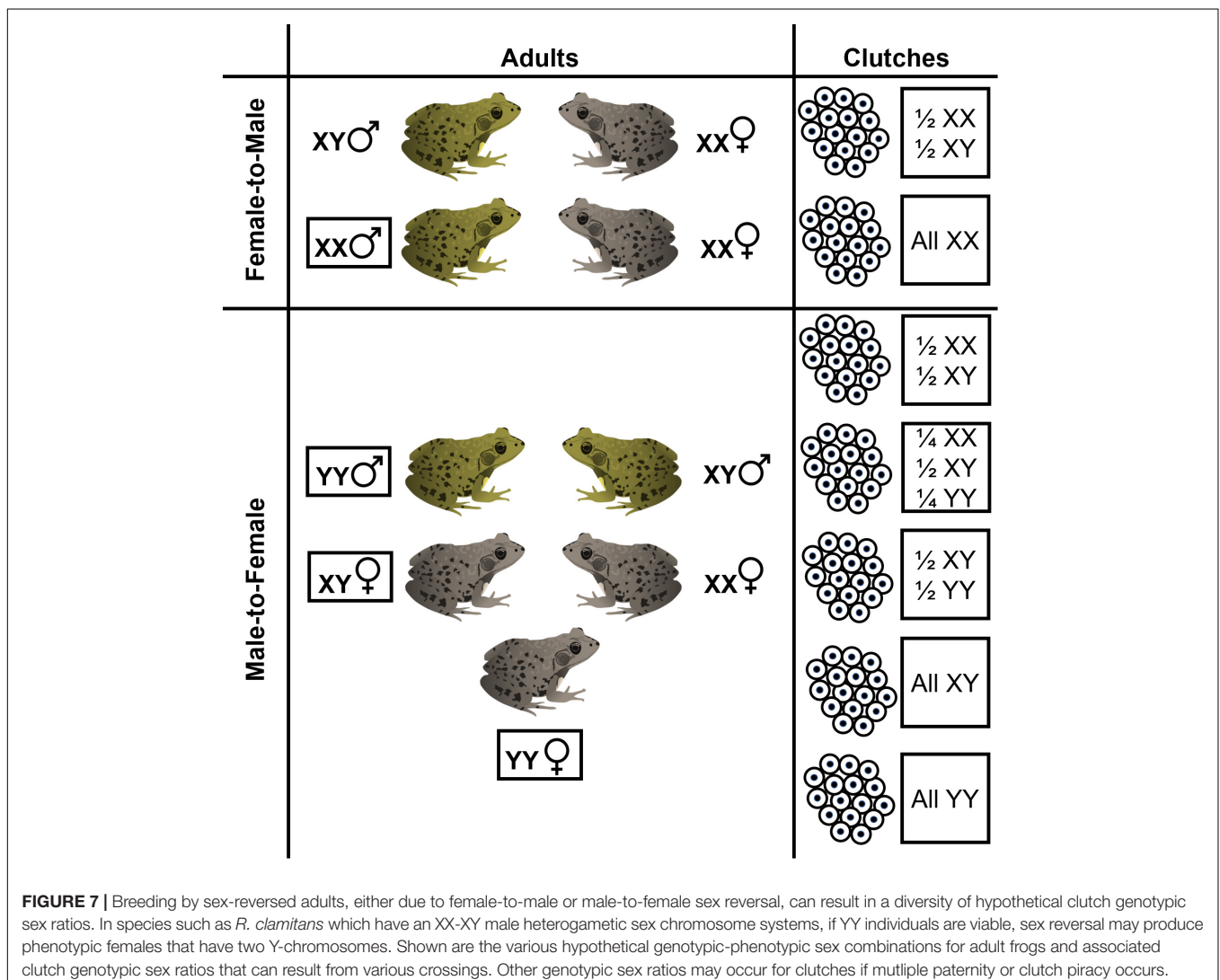
FIGURE 6 | Genotypic and phenotypic sex ratios vary not only among populations but also among years within a population. Horizontal dashed lines indicate a hypothetical 50:50 sex ratio. Here are genotypic and phenotypic sex ratio data from two focal ponds – Forest5 and Septic7 – in the years 2016 and 2018.

sampled across 13 populations in 2012 consistently had male-biased phenotypic sex ratios across multiple forest ponds but equal or female-biased sex ratios in suburban ponds

(Lambert et al., 2015). We found no such association between the environment and sex ratios in metamorphs sampled in 2016. Additionally, due to drought, we had a smaller number

of populations ($n = 6$) available for study. Although this sample size is like what is often used in similar studies (Hayes et al., 2003; Orton and Routledge, 2011; Papoulias et al., 2013), it is half of the sample size of our prior work (Lambert et al., 2015). How yearly differences in environmental conditions influence sex ratios and sex reversal is unclear but documenting interannual variation is likely important for understanding the natural and anthropogenic contributions to wild amphibian sexual demography. Importantly, work in fishes and reptiles suggests that climate change is having and will continue to shape the sexual demography of wild animal populations (Ospina-Alvarez and Piferrer, 2008; Montalvo et al., 2012; Holleley et al., 2015). Additionally, experimental research in amphibians provides unambiguous evidence that temperature can modulate amphibian sex reversal (Lambert et al., 2018; Miko et al., 2021). Given the associations between drought, temperature, sex reversal, and sex ratios observed in *R. clamitans* here, there is a need to understand how climate change will shape sex reversal and population demography in wild amphibians.

We also found that genotypic sex ratios of individual clutches are variable and can deviate from parity. Breeding by sex-reversed individuals could produce a diversity of potential genotypic sex ratios (Figure 7) which, in aggregate, could hypothetically shape cohort sex ratios. Four clutches (two from each pond) had male-biased genotypic sex ratios. The most likely explanation for this pattern is that male-to-female reversed adults successfully breed, although further work is needed to document breeding by sex-reversed frogs. Interestingly, we also found two clutches in our focal forest pond which had female-biased genotypic sex ratios. Mating between only two frogs – a sex-reversed genotypic female and a non-sex reversed female – would produce a clutch with only genotypically female embryos (i.e., all XX sex chromosome genotypes). However, the female-biased clutch genotypic sex ratios we observed were $\sim 2/3$ female, not entirely genotypically female. The only other study, to our knowledge, to assess clutch sex ratios was on European Common Frogs (*Rana temporaria*) and also found several clutches that were $\sim 2/3$ genotypic female (Alho et al., 2010). Alho et al. (2010) interpreted this as a



result of multiple paternity where one father was a sex-reversed XX-male. While the drivers of female-biased (but not entirely female) clutches are unclear, multiple paternity and “clutch piracy” is known to occur in frogs (Laurila and Seppa, 1998; Vietes et al., 2004) and so these sex ratios could potentially be the result of mating between a sex-reversed genotypic female, a sex-concordant male, and a sex-concordant female. Our individual clutch genotypic sex ratios provide suggestive evidence that sex-reversed frogs breed in the wild which may skew individual clutch and entire cohort genotypic sex ratios at fertilization (**Figure 1**). Direct observations are still needed to confirm whether sex-reversed adults successfully reproduce and whether this breeding can sufficiently skew entire cohort genotypic sex ratios. We note that because our clutch samples represent field-collected embryos that were hatched in the lab, it is possible that sex-biased mortality occurred during lab hatching, skewing our inferences of clutch sex ratios. Even so, mortality in our lab-hatched embryos was negligible and likely did not impact our inferences here.

Our work has implications for how we study the role sex variation plays in population, community, and ecosystem biology. The growing recognition that sex reversal in amphibians is common including in environments assumed to be natural and minimally impacted by people – including our observations here – highlights how a binary model of sex (i.e., female vs. male) limits how we study the biology of sex (Alho et al., 2010; Lambert et al., 2019; Nemeshazi et al., 2020). In the case of amphibian sex reversal, we can conceive of at least four sex categories depending on the combination of an individual’s genotypic and phenotypic sex. However, these sex categories may still be incomplete as sexual phenotypes and functions may occur along a gradient rather than discrete categories and sex-reversal may create new emergent behavioral and morphological phenotypes that differ from expectations based on constituent phenotypic or genotypic sexes (Li et al., 2016). Sex ratios can influence intraspecific interactions in a population and sex differences in behavior or morphology can shape predation rates and impacts on community and ecosystem processes (Lode et al., 2004, 2005; Weir et al., 2011; Liker et al., 2014; Fryxell et al., 2015). Given this, sex reversal combined with sex-biased mortality has the potential to influence the role of individuals within populations as well as how populations of different genotypic and phenotypic sex compositions influence the broader biological community and ecosystem properties. There has been an increasing call for biologists to rethink our understanding of sex roles and sexual variation in amphibians may open opportunities to expand this area of inquiry (Ah-King and Ahnesjo, 2013; Monk et al., 2019; Warkentin, 2021).

We document multiple processes that work together to shape phenotypic sex ratios of metamorphosing tadpoles. Particularly noteworthy is the consistent pattern of male-biased mortality between two disparate populations. Sex-biased mortality among pre-metamorphic life stages has, to our knowledge, never been documented before in amphibians and represents a previously-unknown contribution to amphibian population demography. Sex-biased mortality, particularly in the context of sex reversal,

has the potential to shape amphibian ecological and evolutionary dynamics due to the underappreciated influence on both the genotypic and phenotypic sexual makeup of populations. We further highlight how phenotypic sex ratios may not be a reliable proxy for important biological processes. For example, sex-biased mortality could skew *genotypic* sex ratios in one direction, but sex reversal could bring *phenotypic* sex ratios back to parity; superficially this equal sex ratio would appear unremarkable whereas it is in fact shaped by the joint effects of sex-biased mortality and sex reversal. Continued exploration of the processes underlying the sexual demography of wild populations will be essential not only to our general understanding of population biology but to conserving populations and species in the face of global environmental change.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Yale IACUC Protocols 2013-10361 and 2015-10681.

AUTHOR CONTRIBUTIONS

ML, TE, and DS conceived the project idea, contributed to the writing and editing of the manuscript. ML performed field and laboratory procedures and conducted statistical analyses. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.756476/full#supplementary-material>

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