

Research



Cite this article: Giery ST, Zimova M, Drake DL, Urban MC. 2021 Balancing selection and drift in a polymorphic salamander metapopulation. *Biol. Lett.* **17**: 20200901. <https://doi.org/10.1098/rsbl.2020.0901>

Received: 21 December 2020

Accepted: 17 March 2021

Subject Areas:

evolution, ecology

Keywords:

Ambystoma maculatum, eco-evolutionary dynamics, population size, historical resurvey, microgeographic variation, contemporary evolution

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Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.5359383>.

Evolutionary biology

Balancing selection and drift in a polymorphic salamander metapopulation

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Understanding how genetic variation is maintained in a metapopulation is a longstanding problem in evolutionary biology. Historical resurveys of polymorphisms have offered efficient insights about evolutionary mechanisms, but are often conducted on single, large populations, neglecting the more comprehensive view afforded by considering all populations in a metapopulation. Here, we resurveyed a metapopulation of spotted salamanders (*Ambystoma maculatum*) to understand the evolutionary drivers of frequency variation in an egg mass colour polymorphism. We found that this metapopulation was demographically, phenotypically and environmentally stable over the last three decades. However, further analysis revealed evidence for two modes of evolution in this metapopulation—genetic drift and balancing selection. Although we cannot identify the balancing mechanism from these data, our findings present a clear view of contemporary evolution in colour morph frequency and demonstrate the importance of metapopulation-scale studies for capturing a broad range of evolutionary dynamics.

Introduction

The growing recognition that evolutionary processes can occur on contemporary timescales and microgeographic scales compels the need to understand how evolution affects ecology [1–3]. In particular, this realization suggests that ecological and evolutionary processes interact in determining trait distributions, species interactions, population dynamics and ecosystem processes [4,5]. Oftentimes, ecological patterns cannot be fully explained without recourse to underlying evolutionary dynamics [1,6–8].

Concurrently, historical resurveys have emerged as an invaluable approach to understanding ecology and evolution [9–12]. Resurveys can expose dominant ecological processes underlying variation in population abundances [13,14], community composition [15,16] and geographic range [17] and can reveal phenotypic and genetic shifts in wild populations [18–22]. Knowing how and why traits change can provide insights into the fate of wild populations facing rapid global change [23,24]. However, if relegated to single or large populations, historical surveys might be biased in their revelations about underlying evolutionary mechanisms, particularly those operating in small populations such as drift.

Here, we take advantage of a historical survey (1990–1991) to assess contemporary evolution in a spotted salamander (*Ambystoma maculatum*) metapopulation. The focal trait is a visible polymorphism in egg mass coloration (figure 1*a,b*). Because microevolution has been demonstrated in spotted salamander behaviour, morphology and physiology [25–31], we resurveyed this metapopulation to evaluate if morph frequency had evolved over three decades. Based on natural history and theory, we generated a series of *a priori*

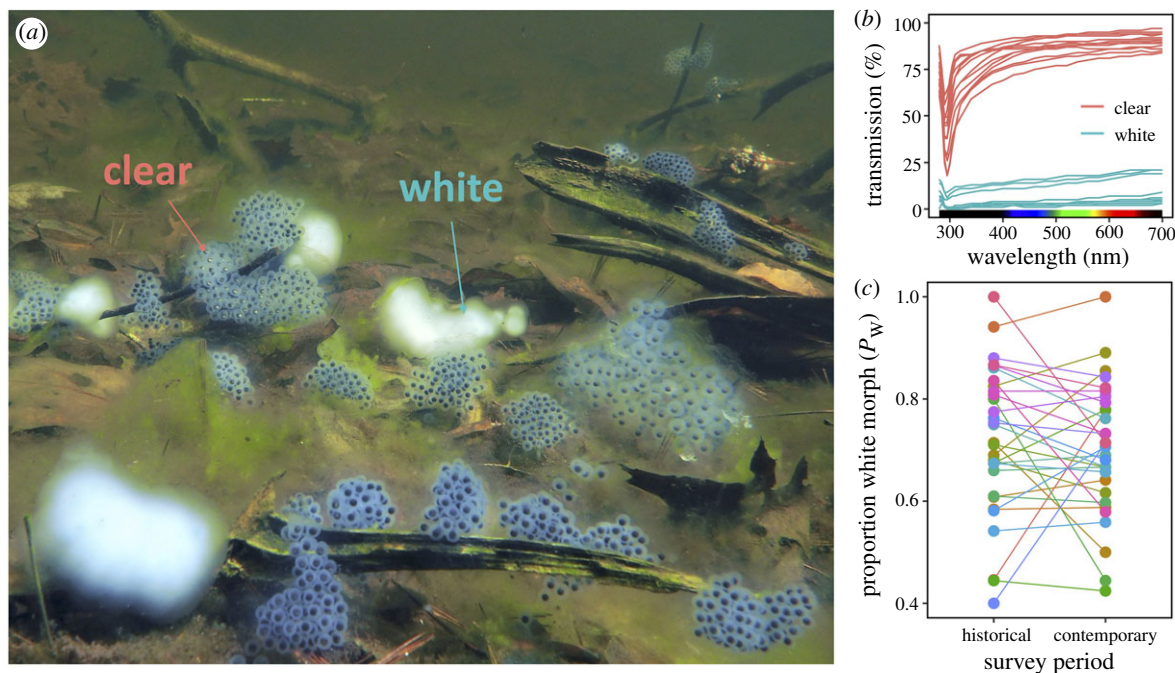


Figure 1. (a) Clear and white morphs of spotted salamander egg masses on the bottom of a temporary pond. (b) Colour variation is discrete, illustrated here by morph-specific transmission of incident light (280–700 nm) through 1 cm of jelly. (c) Morph frequency varied among populations and across time, exemplified by our resurvey of a spotted salamander metapopulation in central Pennsylvania. Each pair of coloured points linked by a line ($n = 31$) illustrates the varied direction and magnitude of population-level morph frequency change between surveys. Photo: Mark C. Urban.

predictions to identify possible modes of evolution: directional selection due to environmental change, balancing selection and genetic drift (table 1).

Methods

Study system

The focal metapopulation is located in 70 km² of broadleaf forest in central Pennsylvania, USA (electronic supplementary material, appendix SI). Although terrestrial as adults, spotted salamanders depend on small, isolated wetlands for reproduction [41]. Adult salamanders migrate to breeding ponds in early spring to mate and lay eggs. Each female oviposits one or more globular clusters called egg masses which include up to several hundred eggs encased in jelly (figure 1a) [42]. After several months, larvae metamorphose and leave breeding ponds [41].

Spotted salamander egg masses are dimorphic in coloration (figure 1a). The clear morph is transparent whereas egg jelly proteins in the white morph [43,44] strongly attenuate light, rendering it almost opaque (figure 1b). For example, at its centre a clear egg mass with a radius of 3 cm receives 68% of incident visible light (400–700 nm). A white egg mass of the same dimensions receives 0.06% (electronic supplementary material, appendix SII). Such striking phenotypic differences make morph classification highly repeatable across surveys and surveyors (intraclass $r = 0.96$; electronic supplementary material, appendix SIII).

Polymorphic and monomorphic populations are found range-wide [43–52]. What, if any, selective agents drive this spatial variation is unresolved [44,48,49,51–55]. The original study of this metapopulation by Ruth [56] suggested a causative link between cation concentration and morph frequency. But while pondwater chemistry appears to have consistent explanatory power [54,56], a mechanistic link with morph frequency remains elusive, as does strong support for competing hypotheses: predation, oxygen limitation and ultraviolet radiation [49,53,55]. Although not conclusive, coloration is likely under genetic control: ovisac anatomy varies by polymorphism [43],

individual females lay the same egg mass morph through time [44], and morph-specific protein profiles are conserved across 1600 km [43,56]. Further, the demonstration of complementary patterns of morph-specific mortality and microgeographic variation is consistent with evolution by natural selection [48].

Data collection and analysis

Ruth [56] documented egg polymorphisms and water chemistry in 34 ponds in Rothrock State Forest in central Pennsylvania. We relocated 31 of them from annotated maps [57] and resurveyed them in April 2020 following original methods (electronic supplementary material, appendix SI: table S1). Briefly, one observer (STG) slowly waded ponds, counting each morph. Prior to each survey, the observer measured pH with an Oakton PCTS 35 probe and collected water for dissolved organic carbon (DOC) analysis [58]. DOC and pH were used as proxies for cation concentrations and environmental change: DOC covaries with sodium ($r = -0.54$, $p < 0.05$), and pH covaries with calcium, potassium and magnesium ($r > 0.6$, $p < 0.05$; electronic supplementary material, appendix SIV).

To assess ecological change since initial surveys, we compared population size (egg mass counts), DOC and pH from the two time periods (historical: 1990–1991; contemporary: 2020). The effect of survey period (t) on population size and DOC was tested with a linear mixed model with random intercepts for ‘population’ effects. Prior to analysis of population size, egg mass counts and DOC data were log-transformed to meet model assumptions (Shapiro–Wilk’s $W = 0.98$, $p = 0.2187$; $W = 0.98$, $p = 0.2419$). Acidity data did not fit Gaussian, lognormal, Poisson or gamma distributions, so the difference between time periods was evaluated with a pairwise Wilcoxon rank-sum test.

We next examined predictions in table 1 and followed model structures articulated there. Unless otherwise noted, all analyses were conducted in R v. 4.0.3 [59] using quantreg [60] and lme4 [61]. All data can be accessed in Dryad [62].

To compare morph frequencies between historical and contemporary surveys, we used a binomial generalized linear mixed model to test for metapopulation-wide shift in the proportion of

Table 1. Hypothesized modes of evolution and associated predictions based on differential effects of survey period (t), environment (e), population size (N) and initial morph frequency (P_{W90}) on morph frequency in 2020 (P_{W20}). Linear models used to evaluate predictions are also formulated: * indicates a significant effect without a specified direction, + and – indicate positive and negative effects, respectively, and ‘n.s.’ indicates no effect.

hypothesis	prediction	survey period (t)	environment change (Δe)	population size (N)	resurvey white morph (P_{W90})	reference(s)
frequency-independent selection	metapopulation-wide shift in mean morph frequency	$P_W \sim t$	*			[32]
	correlated change in morph frequency and environment	$\Delta P_W \sim \Delta e$	*			
	change in morph frequency is greater in large populations	$\Delta P_W \sim N$		+		[33–35]
	change in morph frequency due to environmental change is greater in large populations	$\Delta P_W \sim \Delta e + N$	*	+		[33–35]
balancing selection	morph frequency increases if historically uncommon	$P_{W20} \sim P_{W90}$			+	[36–38]
	no metapopulation-wide shift in morph frequency	$P_W \sim t$	n.s.			[32]
	absolute difference in morph frequency is greater in small populations	$ \Delta P_W \sim N$		–		[38–40]

white (P_W) egg masses with survey period (t) as the fixed effect and random intercepts for each population. Unless specified, we used linear regression for the remaining analyses. Given overlapping generations and the potential for skipped reproductive years in spotted salamanders, we estimated population size as the mean number of egg masses (N) observed across all surveys [63]. To characterize the change in environmental variables (Δe) and morph frequency (ΔP_W), we subtracted each population’s historical values (P_{W90}) from contemporary ones (P_{W20}). To test for balancing selection, we assessed the relationship between morph frequency in the historical period with the contemporary period: $P_{W20} \sim P_{W90}$. In this format, a slope (b) of less than 1 indicates that a balancing mechanism such as negative frequency-dependent selection could be stabilizing morph frequencies across the metapopulation [36,37,64,65].

If drift is driving morph evolution, frequency variance should increase with increasing population size. We used quantile regression, which tracks how the variance distribution of the mean varies, to analyse how the variance of absolute changes in morph frequencies ($|\Delta P_W|$) changed with population size. We also compared observed morph frequencies with Monte Carlo simulations designed to demonstrate sample size effects on drift. We simulated drift for each population, parameterizing each with corresponding field-collected data (initial proportion of white egg masses: P_{W90} , and population size: N). Briefly, samples of size N were drawn from a binomial distribution with replacement for five generations. Initial binomial probabilities were set to P_{W90} . Subsequent generations were initialized with frequencies from preceding iterations to produce a random walk in time. Each sampling event was replicated 100 times per population and included five generations, assuming a 6-year generation time [66–68] (additional detail in electronic supplementary material, appendix SIV). To compare simulated and observed morph frequency change, we analysed the simulated data as we did for the observed data.

Results

Overall, the metapopulation was stable across three decades. No populations became extirpated naturally, and population sizes were similar between survey periods at a mean of 88 egg masses per pond ($F_{1,51.5} = 1.57$, $p = 0.287$). Metapopulation morph frequency was also similar between surveys ($P_{W90} = 0.707$, $P_{W20} = 0.701$; $F = 0.365$, $p = 0.546$). Finally, biogeochemistry did not vary over time (pH = 4.6, $p = 0.32$; DOC = 3.95 mg l⁻¹, $F_{1,56.7} = 0.422$, $p = 0.519$; electronic supplementary material, appendix SI: figure S1)

Although the aggregate view of the metapopulation revealed no net change, morph frequencies in some populations increased while others decreased (ΔP_W : mean = -0.02 , range -0.36 – 0.33 ; figure 1c). Population size (N) did not explain observed morph frequency changes ($F_{1,29} = 0.909$, $p = 0.348$), nor did environmental change (Δ pH: $F_{1,29} = 0.588$, $p = 0.449$; Δ DOC: $F_{1,29} = 0.666$, $p = 0.421$; electronic supplementary material, appendix SI: figure S2). Including population size (N) in environmental change models did not alter the results (Δ pH: $F_{2,28} = 0.005$, $p = 0.967$; Δ DOC: $F_{2,28} = 0.162$, $p = 0.691$).

However, as predicted by balancing selection, we found a slope significantly less than 1 for the positive relationship between historical and contemporary morph frequencies ($b = 0.39$, 95% CI 0.09–0.69, $F_{1,29} = 7.1$, $p = 0.013$; figure 2a). To control for a possible effect of population size, we ran the same model again with N as a covariate; the results were similar ($b = 0.40$, 95% CI 0.09–0.71, $F_{2,28} = 6.9$, $p = 0.014$).

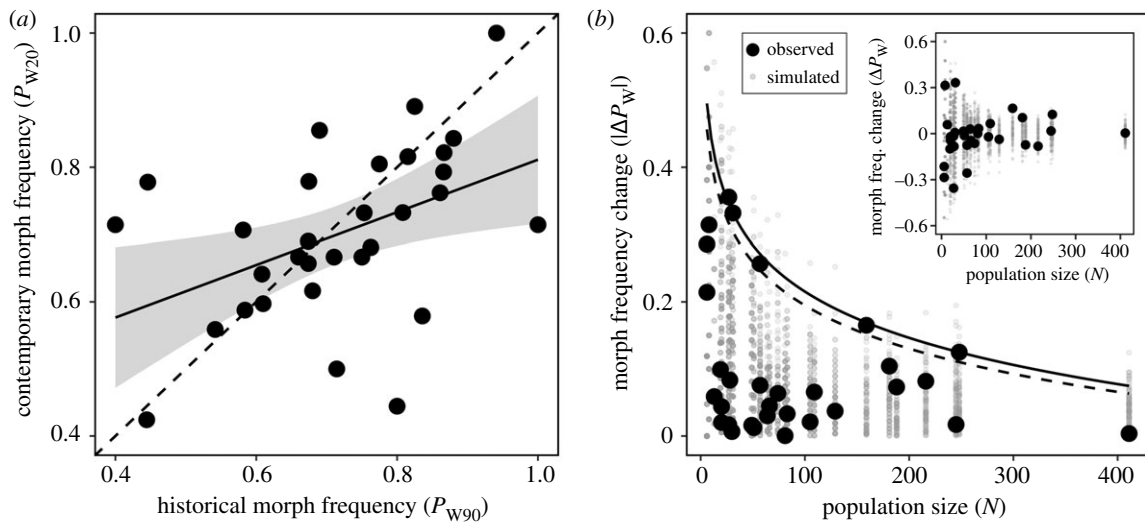


Figure 2. Evidence for multiple modes of contemporary evolution in a spotted salamander metapopulation. (a) A slope of less than 1 ($b = 0.39$) indicates a balancing mechanism such as negative frequency-dependent selection is stabilizing morph frequencies by driving high frequencies lower, and low frequencies higher. The dashed line indicates the 1:1 slope, where no change in frequency occurs between surveys. (b) There was no metapopulation-level difference in morph frequency between periods ($\Delta P_W = -0.017$ and -0.002 for observed and simulated data, respectively; inset). Using absolute change in morph frequency ($|\Delta P_W|$), quantile regression (95th quantile) fitted to observed data (solid line) indicates a population size effect driven by large-magnitude change in small populations. Observed data are a good qualitative match to a Monte Carlo simulation of evolution by drift alone; depicted here by quantile regression (95th quantile, dashed line) through the endpoints of simulated evolution by drift.

Also, as predicted by drift, we found that morph frequency change ($|\Delta P_W|$) was greater and more variable in small populations ($\tau = 0.95$, $t = -2.69$, $p = 0.0118$; figure 2b). Further supporting a role for drift, observed data fell within the 95% confidence intervals of the slope and intercept describing the 95th and 90th quantiles of the simulated drifting metapopulation (electronic supplementary material, appendix S1: table S2).

Discussion

We found no evidence of changes in metapopulation size, population size, biogeochemistry or morph frequencies between surveys. A closer inspection, guided by mechanistic predictions in table 1, indicated that genetic drift and balancing selection were ongoing during the three decades between surveys (figure 2). Our findings match a broad expectation that contemporary evolution is a multifarious process, in which the relative importance of drift and selection depends on population size [32,69–75].

This interplay is crucial for understanding the evolution and persistence of polymorphisms [38,76–79] and the conservation of small populations [32,80–82]. The observed, large-magnitude frequency change in small populations ($N < 100$) is consistent with contemporary evolution by drift—as our simulation demonstrates (figure 2b). However, our test for balancing selection (figure 2a) coupled with the persistence of the polymorphism in populations of all sizes suggests a broad influence of balancing mechanisms. Previous work suggested that spatial heterogeneity in selection could stabilize this polymorphism (e.g. [48,54]), but moderate-to-strong negative frequency-dependent selection could also overcome drift (electronic supplementary material, appendix SVI). For example, given that half of the populations in this metapopulation exceed 57, a selection differential greater than 0.018 would be required to stabilize the polymorphism in the majority of populations [83,84], which seems possible given

evidence for strong selection and evolutionary differences on other traits in this system [26–29]. Ultimately, our results provide support for one or more balancing mechanisms stabilizing morph frequency around an equilibrium frequency—presumably, the observed aggregate mean ($P_W \approx 0.7$; figure 2a). As a caveat, although our results are consistent with metapopulation-wide balancing mechanisms such as negative frequency-dependent selection, we rely solely on two time points spanning multiple generations. More robust inferences could be gained from data collected over shorter intervals.

To better understand this system, future work should confirm the genetic basis for jelly coloration, refine population size effects on drift–selection balance, experimentally probe potential balancing mechanisms [85,86], and examine the possibility that frequency variation can drive eco-evolutionary feedbacks [87–90]. Finally, although balancing selection could stabilize morph frequencies locally, the identity of the possible stabilizing selection on equilibrium morph frequency remains unknown. Given widespread evidence for clinal variation in polymorphism frequencies in other systems [91–93], regional differences in selection seem likely.

When combined with fine-scaled ecological data and replicate populations, historical resurveys can provide unique insights into evolutionary processes in nature. In this case, we leveraged metapopulation-scale resurveys to illustrate how contemporaneous drift and selection can drive microgeographic variation in a poorly understood polymorphism. Crucially, our ability to detect these dynamics depended on including the many small populations that constitute this metapopulation ($N < 100$ for most populations). Although populations of 1000 are often featured in studies (e.g. [51,68]), small populations are much more typical (e.g. [94]). For example, a survey of 405 breeding ponds (total $N = 29\,598$ egg masses) across Pennsylvania found that the median number of egg masses per pond was 33, with 78% of ponds having fewer than 100 (electronic supplementary material, appendix SVII). Given what we learned from our resurvey, drift is likely a significant evolutionary process in these

metapopulations, and for spotted salamanders in general. However, the metapopulation-wide influence of drift could be substantially under-represented if evolutionary dynamics are modelled on rare, large 'charismatic' populations.

Ethics. This research was permitted by the Pennsylvania Fish and Boat Commission (permit no. 2020-01-0102), the Pennsylvania Game Commission (permit no. 48892), and the Pennsylvania Department of Conservation and Natural Resources Bureau of Forestry (permit no. SFRA-2005). The Pennsylvania State University Institutional Animal Care and Use Committee does not regulate early stage, pre-hatching amphibian embryos. To minimize impacts to wild populations, we followed the American Society of Ichthyologists and Herpetologists guidelines for use of live amphibians in research, and disinfected field equipment between surveys to minimize the risk of spreading amphibian pathogens.

Data accessibility. Data are available in the Dryad Digital Repository: <https://doi.org/10.5061/dryad.sf7m0cg5x> [62].

Authors' contributions. S.T.G. designed the study and collected the data. All authors made significant contributions to data analysis and writing, gave final approval for publication and agree to be held accountable for the work performed herein.

Competing interests. We declare we have no competing interests.

Funding. M.C.U. and D.L.D. were supported by NSF award DEB-1555876. M.Z. was supported by a University of Michigan Institute for Global Change Biology fellowship. S.T.G. was supported by an Eberly Research Fellowship of the Eberly College of Science at The Pennsylvania State University.

Acknowledgements. B. Assis, E. Cheng, J. Kolbe, T. Langkilde, N. Lemoine and C. Nadeau provided valuable feedback on early versions of the manuscript. Thank you to Z. Lai for laboratory access and to C. Rowe for assistance reconstructing the historical dataset.

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