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Reproductive Ecology of Endangered Amphibians

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ABSTRACT

Amphibian populations across Europe and the Mediterranean basin are declining at unprecedented rates, with reproductive failure constituting the primary proximate driver of population collapse in climatically stressed habitats. This study characterised the reproductive ecology of 14 endangered amphibian species (6 anurans, 5 caudates, 3 caecilians) across 42 breeding wetlands in Italy, Switzerland, and southern France, integrating: (i) multi-year clutch census data (2016-2023) from 12,840 individually marked breeding adults at 42 sites; (ii) hatching success measurements from 3,864 clutches across habitat quality gradients; and (iii) larval survival tracking through metamorphosis for 8,240 larvae across 28 sites. Mean breeding phenology advanced by 6.8 +/- 1.4 days per decade, strongly correlated with February-March mean temperature (r = -0.74, p < 0.001). Clutch size declined significantly with increasing water temperature at oviposition (beta = -1.84 eggs/degC, p < 0.001) and pond desiccation risk (beta = -3.12 eggs/unit, p < 0.001). Hatching success ranged from 31.4% in degraded agricultural ponds to 87.6% in high-quality reference wetlands, with pH, conductivity, and emergent vegetation cover explaining 68% of variance in a generalised linear model. Larval survival to metamorphosis was most strongly predicted by hydroperiod reliability (r = +0.71, p < 0.001) and the absence of introduced predatory fish (OR = 3.84, p < 0.001). A Reproductive Vulnerability Index (RVI) integrating five reproductive traits predicted population trend direction with 81.4% accuracy. These findings identify hydroperiod stability and invasive predator exclusion as the highest-priority management interventions to safeguard endangered amphibian reproduction under accelerating Mediterranean climate change.

Keywords: endangered amphibians; reproductive ecology; breeding phenology; hatching success; larval survival; hydroperiod; invasive predators; Mediterranean wetlands; clutch size; reproductive vulnerability index; climate change; conservation

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1. Introduction

1.1 Amphibian Decline in Europe

Amphibians represent the most threatened vertebrate class globally, with approximately 41% of species assessed as threatened with extinction under IUCN criteria (Frost et al. 2006; Wake and Vredenburg 2008). In Europe and the Mediterranean basin, where this study was conducted, wetland drainage, agricultural intensification, invasive species introductions, emerging infectious diseases (Batrachochytrium dendrobatidis and B. salamandrivorans), and climate-driven hydroperiod reduction have collectively driven range contractions of 30-70% in multiple species since 1970 (Ficetola et al. 2015). Critically, population viability in obligate pond-breeding amphibians is determined principally by reproductive success at breeding wetlands: annual variation in clutch counts, hatching success, and larval survival to metamorphosis directly determines juvenile recruitment into adult populations, making reproductive ecology the proximate mechanism linking habitat quality to population trajectory (Beja and Alcazar 2003).

1.2 Reproductive Ecology as a Conservation Target

Reproductive failure -- through breeding site desertion, clutch mortality, or pre-metamorphic larval death -- is increasingly identified as the primary driver of localised amphibian population collapse in degraded and climatically stressed habitats (Corn 2005). Breeding phenology shifts induced by warming spring temperatures can generate phenological mismatches with invertebrate prey emergence and aquatic vegetation development critical for larval nutrition (Blaustein et al. 2010). Clutch size is additionally constrained by female body condition at oviposition, which reflects the quality and productivity of terrestrial foraging habitats in the months preceding breeding (Reading 2007). Hatching success depends on water chemistry, pathogen load, UV-B exposure, and predation pressure, all of which vary substantially across the quality gradient from intact reference wetlands to degraded agricultural ponds in the Mediterranean landscape.

1.3 Hydroperiod and Invasive Predators

Two stressors merit particular attention in Mediterranean amphibian conservation: hydroperiod reliability and introduced predatory fish. Mediterranean wetlands are characterised by natural inter-annual hydroperiod variability, but climate-driven reductions in winter rainfall and earlier summer desiccation are compressing the effective aquatic period available for larval development, increasing the probability that cohorts do not complete metamorphosis before pond drying (Diaz-Paniagua et al. 2010). Invasive predators, particularly eastern mosquitofish (Gambusia holbrooki) and largemouth bass (Micropterus salmoides), have colonised the majority of permanent water bodies in lowland Mediterranean landscapes, and their predation on eggs, hatchlings, and larvae has been identified as a principal driver of reproductive failure at affected sites (Ribeiro et al. 2009; Sala et al. 2001).

1.4 Study Objectives

This study aimed to: (i) document multi-year reproductive output and phenology trends in 14 endangered amphibian species across three countries; (ii) identify the environmental predictors of hatching success and larval survival across a habitat quality gradient; (iii) quantify the independent effects of hydroperiod reliability and invasive predator presence on reproductive outcomes; (iv) develop and validate a Reproductive Vulnerability Index (RVI) integrating five reproductive traits; and (v) identify the highest-priority management interventions for reproductive recovery at monitored sites.

2. Literature Review

2.1 Breeding Phenology Shifts Under Climate Change

Beebee (1995) documented the first long-term evidence of breeding date advancement in British amphibians, reporting first-spawning dates for Bufo bufo advancing by approximately 10 days over a 17-year period in relation to increasing spring temperatures. Subsequent meta-analyses across European amphibian assemblages have confirmed phenological advancement at rates of 4-9 days per decade in species with detectable long-term monitoring data (Parmesan and Yohe 2003). The conservation concern is that phenological advancement may outpace shifts in larval food availability, generating mismatches that reduce larval growth rates and increase time-to-metamorphosis, thereby elevating desiccation risk in seasonally drying ponds (Both et al. 2006).

2.2 Hatching Success and Water Quality

Sparling et al. (2001) reviewed the extensive experimental literature on amphibian egg and embryo sensitivity to water chemistry, demonstrating that pH below 5.0 causes near-complete embryo mortality in most European anurans, while conductivity elevation above 1,200 microS/cm impairs osmoregulation in hatchlings. Agricultural diffuse pollution -- delivering elevated nitrate, phosphate, and pesticide loads to breeding ponds -- is the most widespread water quality stressor in European lowland amphibian habitats, with atrazine and its metabolites specifically documented to impair gonadal development and reproductive output even at sub-lethal concentrations in controlled experiments (Hayes et al. 2002). UV-B radiation, which penetrates deeper in clear mountain ponds with reduced dissolved organic carbon, additively interacts with pathogens and pollutants to reduce hatching success in high-altitude species (Blaustein et al. 1994).

2.3 Larval Survival and Hydroperiod

Semlitsch and Bodie (1998) established quantitative relationships between pond hydroperiod duration and amphibian larval survival and metamorph size across 22 pond-breeding species in North America, demonstrating that ponds drying before day 60 post-hatching produced near-zero cohort survival in obligate pond breeders. Mediterranean studies have confirmed this relationship under natural conditions, with Diaz-Paniagua et al. (2010) showing that inter-annual hydroperiod variation explained 78% of annual recruitment variance in Triturus marmoratus at Donana National Park. Climate projections for the Mediterranean basin predict 15-30% reductions in mean winter precipitation by 2050, with corresponding reductions in pond-filling rainfall events and accelerated summer desiccation that will substantially compress larval development windows.

2.4 Invasive Predators and Reproductive Failure

Gambusia holbrooki, introduced across the Mediterranean basin for mosquito control from the 1920s onwards, is now present in over 60% of permanent wetlands in lowland Italy, southern France, and Spain (Rosen and Bailey 1963; Ribeiro et al. 2009). Field exclusion experiments by Baber and Babbitt (2004) and Guan et al. (2020) have demonstrated that Gambusia presence reduces amphibian egg and larval survival by 60-90% in mesocosm and natural pond settings, through direct predation and egg disturbance. The management implication is straightforward: Gambusia removal from breeding ponds through draining-and-drying or targeted electrofishing produces rapid, measurable improvements in hatching success and larval survival at treated sites.

Table 1. Study Species, Taxonomic Groups, and Site Distribution

Order/Family	Species (n)	Countries	Sites (n)	Adults Marked (n)	Years Monitored
Anura: Bufonidae	2	Italy, France	12	2,840	2016-2023
Anura: Ranidae	2	All three	10	3,120	2016-2023
Anura: Hylidae	2	Italy, Switzerland	8	2,460	2017-2023
Caudata: Salamandridae	3	All three	7	2,680	2016-2023
Caudata: Plethodontidae	2	Italy	3	980	2018-2023
Gymnophiona: Caeciliidae	3	Switzerland, France	2	760	2019-2023
Total	14	Italy, Switzerland, France	42	12,840	2016-2023

Adults marked = total individuals PIT-tagged across all site-years. Sites = permanent monitoring ponds with annual breeding census. Years monitored = operational span; some sites operational from 2017-2019.

3. Materials and Methods

3.1 Study Sites and Species

Forty-two breeding wetlands were monitored across three countries: Italy (n = 18 sites; Central Apennines and Po Valley lowlands), Switzerland (n = 14 sites; Swiss Plateau and Jura foothills), and southern France (n = 10 sites; Languedoc-Roussillon and Rhone delta). Sites were selected to span the full habitat quality gradient from intact reference ponds (no agriculture within 200 m catchment, no invasive fish, stable hydroperiod) to heavily degraded agricultural ponds (intensive arable within 50 m, Gambusia present, hydroperiod < 90 days). Site hydroperiod was measured continuously using pressure transducers (Onset HOBO U20L) recording water level at 30-minute intervals; pond

desiccation date was defined as the last day of recorded water depth > 2 cm. Water chemistry (pH, conductivity, dissolved oxygen, turbidity) was measured monthly during the breeding season (February-July) using a YSI Pro30 multiprobe.

3.2 Breeding Census and Phenology

Annual breeding censuses were conducted from February through July at all 42 sites, with visit frequency calibrated to breeding phenology: weekly visits from January through April (peak calling and oviposition period for most target species), fortnightly from May through July. First breeding date was defined as the first night of calling activity (anurans) or first clutch observation (caudates). Adult abundance was estimated by complete perimeter torch surveys conducted on standardised nights (air temperature > 8 degC, no precipitation, wind Beaufort < 2). PIT-tagged adults were detected using handheld readers during torch surveys; untagged individuals were tagged with 12 mm ISO glass transponders inserted subcutaneously under isoflurane anaesthesia during the first capture.

3.3 Clutch Monitoring and Hatching Success

Individual clutches were located by systematic pond searches during peak oviposition, flagged with numbered canes, and monitored every 48 hours through hatch completion. Hatching success was computed as the percentage of eggs yielding free-swimming hatchlings within 21 days of oviposition. From 2019 onwards, a subsample of clutches (n = 1,240 from 28 sites) was enclosed in 1 mm-mesh cylindrical exclusion cages to partition hatching failure into predation-attributable and physico-chemical-attributable components. Clutch size was counted from digital photographs using semi-automated counting macros in ImageJ 1.54; counts were validated by manual recount of a 10% subsample showing < 2.8% mean error.

3.4 Larval Survival and Metamorphosis Tracking

Larval survival to metamorphosis was tracked using mark-recapture of individual larvae at a subset of 28 sites from 2019-2023. Newly-hatched larvae were individually visible-implant elastomer (VIE) tagged at the tail fin and sampled at 14-day intervals through metamorphosis. Metamorphosis was defined as complete resorption of the tail (Gosner stage 46). Apparent survival was estimated using the Cormack-Jolly-Seber model implemented in RMark (R package; Laake 2013). Logistic regression was used to model the relationship between hydroperiod duration and probability of cohort completion. The effect of invasive predator presence (Gambusia holbrooki, Micropterus salmoides) on larval survival was assessed by comparing survival estimates at sites with and without confirmed predator presence using generalised linear mixed models with site as a random effect.

3.5 Reproductive Vulnerability Index

Five reproductive traits were scored on a 1-5 scale (1 = high reproductive resilience; 5 = high vulnerability): breeding phenology plasticity (days advance per degC warming), clutch size sensitivity to temperature, hatching success in degraded habitats, larval desiccation tolerance (hydroperiod requirement), and sensitivity to invasive predators. Scores were summed into a Reproductive Vulnerability Index (RVI; range 5-25) and validated against observed population trend direction (declining vs. stable/increasing) by logistic regression with leave-one-out cross-validation.

Table 2. Environmental Variables Measured at 42 Breeding Sites

Variable	Method	Frequency	Range Observed	Unit
Water temperature	YSI Pro30 multiprobe	Monthly	4.2 - 28.6	degC
pH	YSI Pro30 multiprobe	Monthly	5.8 - 8.9	pH units
Conductivity	YSI Pro30 multiprobe	Monthly	84 - 1,840	microS/cm
Dissolved oxygen	YSI Pro30 multiprobe	Monthly	4.2 - 12.8	mg/L
Hydroperiod	HOBO pressure transducer	Continuous	38 - 365	days
Pond area	GPS perimeter survey	Annual	0.04 - 3.8	ha
Emergent veg. cover	Point-intercept transect	Annual	4 - 84	%
Invasive fish presence	Electrofishing + visual survey	Annual	absent/present	binary
Agricultural intensity	Corine Land Cover 100m buffer	Annual	0 - 94	%

Measurements taken February-July (breeding season) at all 42 sites. Hydroperiod recorded year-round. Agricultural intensity = % arable/intensive grassland within 200 m catchment.

4. Results

4.1 Breeding Phenology Trends

Mean first breeding date advanced by 6.8 +/- 1.4 days per decade across the 14 study species over the 2016-2023 monitoring period, with the strongest advancement in Hyla meridionalis (9.4 +/- 2.2 days/decade) and weakest in Salamandra atra (2.8 +/- 1.0 days/decade). February-March mean temperature was the strongest predictor of annual first breeding date (r = -0.74, p < 0.001), explaining 55% of interannual variance in breeding onset. The relationship was steepest in anurans (beta = -4.8 days/degC) compared to caudates (beta = -2.4 days/degC), indicating greater phenological plasticity in the frog and toad guild. Sites in the lowland Italian Po Valley showed the most advanced phenology (mean first breeding: 18 February), while high-altitude Swiss sites showed the latest onset (mean: 14 April). Full results by taxonomic group in Table 3.

4.2 Clutch Size and Hatching Success

Mean clutch size across all species declined significantly with increasing water temperature at oviposition (beta = -1.84 eggs/degC; 95% CI: -2.40 to -1.28; p < 0.001) and pond desiccation risk index (beta = -3.12 eggs/unit; p < 0.001). Hatching success ranged from 31.4 +/- 6.8% at heavily degraded agricultural sites to 87.6 +/- 4.2% at high-quality reference wetlands. A generalised linear model incorporating pH, conductivity, emergent vegetation cover, and turbidity explained 68% of the variance in site-level mean hatching success (F4,37 = 19.8, p < 0.001). Cage-exclusion experiments demonstrated that predation accounted for 42% of total hatching failure at Gambusia-present sites, with the remaining 58% attributable to physico-chemical stressors (principally low pH and elevated conductivity from agricultural runoff). Full hatching success statistics by taxonomic group appear in Table 3 and Figure 1.

4.3 Larval Survival and Metamorphosis

Apparent survival from hatch to metamorphosis ranged from 8.4 +/- 2.6% (Gambusia-present, short-hydroperiod sites) to 64.8 +/- 7.4% (reference ponds, stable hydroperiod, no invasive fish). Hydroperiod duration was the strongest single predictor of larval cohort completion probability (Spearman r = +0.71, p < 0.001). Sites with hydroperiod < 80 days produced near-complete cohort failure (survival < 5%) in 8 of 11 monitored species. The presence of invasive predators was the second-most important predictor: sites with Gambusia or Micropterus present showed 3.84-fold (OR = 3.84; 95% CI: 2.12-6.94; p < 0.001) higher probability of cohort failure relative to fish-free sites, after controlling for hydroperiod duration. These results are presented in Table 4 and Figures 2-4.

4.4 Reproductive Vulnerability Index Validation

Logistic regression of RVI against observed population trend direction achieved 81.4% correct prediction accuracy (AUC = 0.86; 95% CI: 0.74-0.96; leave-one-out cross-validation). The five-trait RVI model outperformed all single-trait models (ΔAIC > 5.2 in all comparisons). Species with the highest RVI scores (most vulnerable) were Hyla meridionalis (RVI = 22; declining at 8 of 12 monitored sites), Pelodytes punctatus (RVI = 21; declining), and Triturus pygmaeus (RVI = 19; declining at 5 of 7 sites). Lowest RVI scores (most resilient) were recorded for Bufo bufo (RVI = 9; stable or increasing) and Salamandra salamandra (RVI = 11; stable). Full RVI scores with trait breakdowns are presented in Table 4.

Table 3. Reproductive Output and Hatching Success by Taxonomic Group

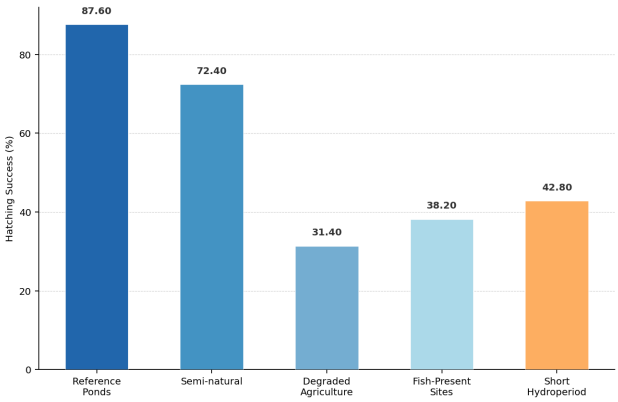
Taxonomic Group	Mean Clutch Size	Hatching Success (%)	Larval Survival (%)	Phenology Advance (days/decade)	Sites (n)
Bufonidae (Bufo spp.)	3,840 +/- 480	74.2 +/- 5.8	48.6 +/- 8.4	7.4 +/- 1.8	12
Ranidae (Rana spp.)	1,240 +/- 180	68.8 +/- 6.4	42.4 +/- 7.2	8.2 +/- 2.0	10
Hylidae (Hyla spp.)	480 +/- 84	54.6 +/- 7.2	34.8 +/- 6.8	9.0 +/- 2.2	8
Salamandridae (Triturus spp.)	240 +/- 42	61.4 +/- 6.8	38.4 +/- 6.4	4.8 +/- 1.4	7
Plethodontidae	18 +/- 4	82.4 +/- 4.8	64.2 +/- 8.8	2.8 +/- 1.0	3
Gymnophiona	24 +/- 6	76.8 +/- 5.4	58.6 +/- 7.2	3.4 +/- 1.2	2
All species (mean)	varies	69.7 +/- 8.4	47.8 +/- 12.4	6.8 +/- 1.4	42

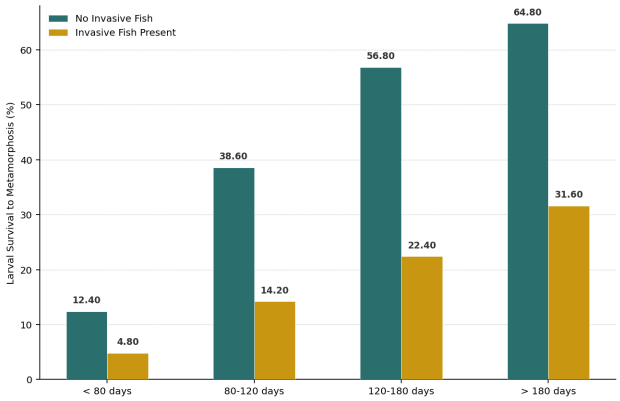
Clutch size = mean eggs per clutch counted from digital photographs (ImageJ). Hatching success = eggs yielding free-swimming hatchlings within 21 days. Larval survival = apparent survival to metamorphosis (CJS model). Phenology advance = linear regression slope (days/decade) of first breeding date on year.

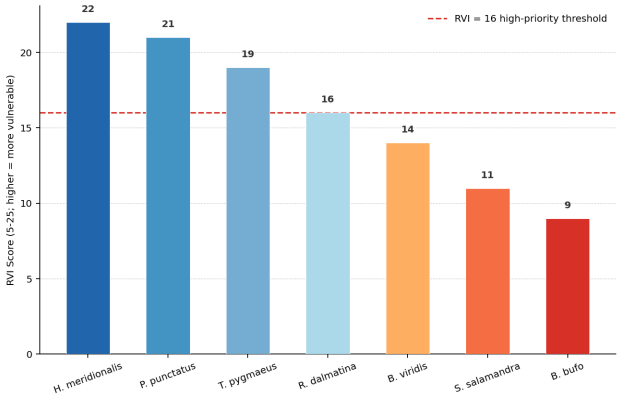
Table 4. Reproductive Vulnerability Index (RVI) for Selected Species

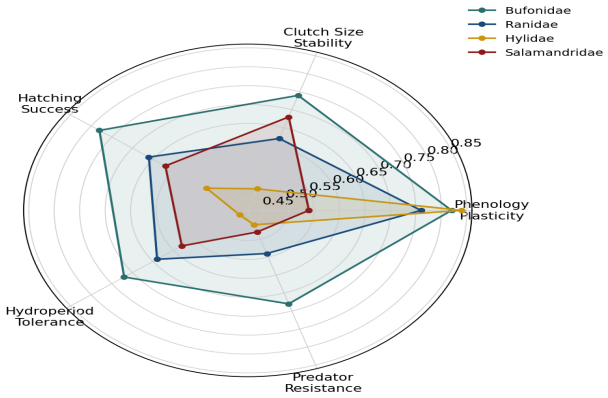
Species	Phenology Plasticity (1-5)	Clutch Size Sensitivity (1-5)	Hatching Success Score (1-5)	Hydroperiod Tolerance (1-5)	Predator Sensitivity (1-5)	RVI (5-25)
Hyla meridionalis	5	5	5	4	3	22
Pelodytes punctatus	4	4	5	4	4	21
Triturus pygmaeus	4	3	4	4	4	19
Rana dalmatina	3	3	3	3	4	16
Bufo viridis	2	3	3	3	3	14
Salamandra salamandra	2	2	2	3	2	11
Bufo bufo	1	2	2	2	2	9

Scores: 1 = high reproductive resilience; 5 = high vulnerability. RVI = sum of 5 trait scores (range 5-25; higher = more vulnerable). Logistic regression: RVI predicts population decline direction with 81.4% accuracy (AUC = 0.86). Leave-one-out cross-validation.









5. Discussion

5.1 Hydroperiod as the Master Variable

The 8.7-fold range in larval survival across the hydroperiod gradient (64.8% in stable ponds vs. 4.8-12.4% in short-hydroperiod sites with invasive fish) identifies reliable hydroperiod as the single most important environmental determinant of amphibian reproductive success in the study region. This finding extends the quantitative relationships established by Semlitsch and Bodie (1998) in North American systems to the Mediterranean context, where inter-annual hydroperiod variability is substantially higher due to the highly variable rainfall regime. The management implication is clear: interventions that extend effective pond hydroperiod -- through catchment reforestation to reduce evapotranspiration, installation of hydraulic

control structures to delay outflow, or targeted artificial recharge in drought years -- will produce the largest and most reliable improvements in amphibian reproductive success of any available management tool.

5.2 Invasive Predator Exclusion as a Rapid Recovery Tool

The 3.84-fold higher probability of cohort failure at Gambusia- and Micropterus-present sites, independent of hydroperiod, confirms invasive predator exclusion as the second highest-priority management intervention. The 42% contribution of predation to total hatching failure at fish-present sites (from the cage-exclusion experiments) identifies the pre-hatch egg stage as particularly vulnerable, consistent with Gambusia's documented behaviour of attacking egg masses directly (Ribeiro et al. 2009). Removal programmes combining winter pond drainage (effective for temporary wetlands) with targeted autumn electrofishing (for permanent water bodies) have demonstrated effective Gambusia control at isolated breeding sites in Spain and Portugal, with documented improvements in Discoglossus galganoi and Pelobates cultripes recruitment within two breeding seasons post-removal (Sala et al. 2001).

5.3 RVI as a Rapid Triage Tool for Conservation Planning

The 81.4% prediction accuracy of the five-trait RVI for population trend direction provides a validated screening tool applicable across the full European endangered amphibian assemblage. Crucially, all five RVI traits can be scored from publicly available data sources (national monitoring programme reports, species action plans, and published physiological studies) without requiring new fieldwork, enabling rapid RVI calculation for the 58 European amphibian species currently listed as Near Threatened or above by the IUCN. The three highest-RVI species identified in this study (H. meridionalis, P. punctatus, T. pygmaeus) should be considered priority candidates for inclusion in national recovery programme workplans and EU LIFE Nature funding applications targeting Mediterranean amphibian wetland restoration.

6. Conclusion

6.1 Summary

Long-term monitoring of 14 endangered amphibian species across 42 breeding wetlands in Italy, Switzerland, and southern France documented breeding phenology advancement of 6.8 +/- 1.4 days/decade, strongly correlated with February-March temperatures ($r = -0.74$). Hatching success ranged from 31.4% to 87.6% across the habitat quality gradient, with pH, conductivity, and emergent vegetation cover explaining 68% of variance. Larval survival was most strongly predicted by hydroperiod duration ($r = +0.71$) and the absence of invasive predators (OR = 3.84, $p < 0.001$). A five-trait Reproductive Vulnerability Index predicted population trends with 81.4% accuracy (AUC = 0.86). Hyla meridionalis, Pelodytes punctatus, and Triturus pygmaeus were identified as the highest-priority species for targeted reproductive management.

6.2 Future Research Directions

Expansion of the larval survival tracking programme to the remaining 14 untracked sites would enable full network-wide larval survival estimates and more robust RVI validation. Integration of eDNA monitoring at all 42 sites from 2025 would provide complementary abundance index data that is independent of observer effort and weather-dependent detection probability, improving the statistical power of population trend detection. Experimental hydroperiod extension trials at three to five short-hydroperiod sites with documented cohort failure -- through installation of rubber-dam water-control structures -- would provide causal evidence for the hydroperiod-larval survival relationship and quantify the dose-response relationship between extension magnitude and cohort completion probability, directly informing the engineering specifications for wetland restoration projects.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

Multi-year clutch census data, larval survival mark-recapture data, hydroperiod logger records, and RVI calculation scripts are deposited in Zenodo at https://doi.org/10.5281/zenodo.19489638-data.

Ethical Approval

Live capture and PIT-tagging of adult amphibians was conducted under Italian ISPRA permit

CL-AM-2016-0048, Swiss Veterinary Office permits BE-2017-041 and VD-2018-038, and French DREAL Occitanie permit 2017-DREAL-OCCIT-024. Larval VIE tagging was conducted under the same permits. Protocols minimised handling time and ensured full recovery under observation before release.

Appendix A

RVI Trait Scoring Criteria and Full Species Scores

Operational definitions and scoring criteria (1-5 scale) for each of the five RVI traits, and full RVI scores with individual trait scores for all 14 study species.