Population ecology of the yellow-bellied toad (*Bombina variegata)* at the northern range margin: Focusing on demography and genetics

von

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„Zur Laichzeit findet eine derartige Ansammlung in dem Weiher daselbst statt, dass das Unkenkonzert den Bewohnern überaus lästig wird. Herr Böcking teilte mir mit, dass man sie dann in Menge fange und ganze Eimer voll mit Kalk begiesse, um sich ihrer zu erwehren.“

aus: Wirbeltierfauna von Kreuznach (Ludwig Geisenheyner)

2018

„Sie sterben, die Frösche, werden still und leise ausgerottet, ohne dass es die Welt kümmert, ein Drittel aller Arten sind ernsthaft bedroht, aber niemand denkt an ihn, den Frosch, der sich durch die Sumpfgebiete unserer Erde bewegt, immer im Kontakt mit dem Wasser, schleimig und unansehnlich, nicht eklig genug, um hässlich zu sein, nicht seltsam genug, um lustig zu sein, nur etwas wunderlich, mit seinem Quaken und seinen Sprüngen, auf der Flucht vor den Menschen.“

aus: Die Geschichte des Wassers (Maja Lunde)

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General introduction

Amphibians are among the most endangered taxa worldwide with about one third of the known species threatened by extinction and more than 40% of the populations considered as declining (IUCN 2019; Stuart et al. 2004). Anthropogenic pressure leading to habitat destruction and fragmentation is a major cause for this trend (e.g., Becker et al. 2007; Gallant et al. 2007). Global warming, pollution, invasive species, UVB radiation and disease pose additional and interacting threats to amphibians (e.g., Blaustein et al. 2011; Collins 2010; Collins & Storfer 2003; Stuart et al. 2004; Wake & Vredenburg 2008). This ongoing worldwide decline has been observed for several decades and mirrors a global biodiversity loss (e.g., Collins 2010; Houlahan et al. 2000).

The yellow-bellied toad (*Bombina variegata*): a European amphibian in decline

An example for a European amphibian species with declining populations is the yellow-bellied toad *Bombina variegata* (Linnaeus, 1758). The range of yellow-bellied toads extends from France in the West and Germany in the North to Greece in the South-East, including great parts of Central Europe and the Balkan region (Gollmann & Gollmann 2012). According to the IUCN Red List of Threatened Species, it is still classified as Least Concern (LC) in Europe (Kuzmin et al. 2009). As the population trend is decreasing, however, it is listed in Annexes II and IV of the Natural Habitats Directive and protected by national law in much of its range (Kuzmin et al. 2009). *Bombina variegata* is still abundant in the eastern and south eastern parts of its range, even though local declines or extinctions were observed (Gollmann & Gollmann 2012). At the northern and western range margin, however, there seems to be serious decline (Gollmann et al. 2012). In northern Italy, the number of *B. variegata* populations is considered stable, while abundance is decreasing in some regions (Barbieri et al. 2004; D'Amen & Bombi 2009). In France, the decline of *B. variegata* dates back to the 19th century and has exacerbated since the 1970ies (Lescure et al. 2011). Yellow-bellied toads underwent a rapid decline in Switzerland as well (Abbühl & Durrer 1993). The main causes are degradation or destruction of habitats, particularly of reproduction ponds (Gollmann & Gollmann 2012). Modified land use led to the fragmentation of former comprehensive *B. variegata* habitats and thus connected populations became isolated (Schlüpmann 1996).

Status of *Bombina variegata* in Germany

About one of third of the world's yellow-bellied toads is found in Germany, thus making the country highly responsible for its conservation (Kühnel et al. 2009). Likewise, *B. variegata* is at its northern range margin in Germany, where its primary habitats, particularly holms of streams and rivers, were destroyed in favour of urbanisation and agriculture. Today, it mainly inhabits secondary, anthropogenic habitats like quarries or military training areas which provide the landscape dynamics that are required for reproduction (Nöllert & Günther 1996). As a sharp decline has been observed over the last decades, *B. variegata* is listed as endangered in the latest national red list and even as extinct (category 0 for Saxony) or as critically endangered in some federal states (category 1 for Lower Saxony, Thuringia and North Rhine-Westphalia) (Kühnel et al. 2009). In Rhineland-Palatinate, *B. variegata* is highly endangered (category 2), as extant populations are often fragmented (Kühnel et al. 2009; Veith 1996a). It is unclear, however, if this red list still mirrors the recent status of *B. variegata* populations in Rhineland-Palatinate, as the latest country-wide assessment dates back to the 1990s (Bitz & Simon 1996; Kühnel et al. 2009). Anthropogenic threats in this region are recultivation of former mining areas, drainage and destruction of wetland, intensification of agriculture and thoroughfares (Bitz 1992). The loss of ponds in and near forests led to the extinction of numerous populations (Veith 1996a). Furthermore, succession poses a serious threat to remaining *B. variegata* populations in Rhineland-Palatinate (Veith 1996a).

A northern distribution centre: the Westerwald region

The low mountain range Westerwald was considered as one of the key areas in Rhineland-Palatinate and one of the northernmost regions extensively inhabited by yellow-bellied toads (Nöllert & Günther 1996). As road and rail networks expanded in the 19th century, this region became an internationally important clay mining area (Schenk 1993). Emerging mines provided secondary habitats for yellow-bellied toads, but the expansion of transport networks certainly contributed to landscape and thus habitat fragmentation. Population declines have been observed in the Westerwald region for more than 40 years (Gruschwitz 1981). However, this region may still function as a basis for (re)distribution and therefore, remaining populations are of great conservation interest. Further research is needed to determine the status quo of extant populations and to develop adequate conservation measures for the persistence of yellow-bellied toads at the northern range margin.

Carry-over effects of larval environment on life history

The conservation of threatened species demands the investigation of their life histories (Atkins et al. 2020). Life-history theory addresses the question how reproductive success of organisms is achieved by evolutionary mechanisms (Stearns 2000). *Bombina variegata* is an excellent model organism to study complex life-history strategies. It spawns in a broad range of breeding ponds and produces metamorphs of high plasticity (e.g., Barandun & Reyer 1997a; Barandun & Reyer 1997b; Di Cerbo & Biancardi 2010; Dittrich et al. 2016; Hantzschmann & Sinsch 2019; Hartel et al. 2007; Kapfberger 1984; Schäfer et al. 2018). According to the life-history theory, amphibians adjust their developmental rate depending on environmental conditions in the larval habitats (Wilbur & Collins 1973). Thus, species inhabiting uncertain environments produce highly plastic offspring (Wilbur & Collins 1973). Environmental conditions experienced in the aquatic habitat may influence the timing of metamorphosis as well as morphological and behavioural features of the terrestrial metamorph, causing variation in adult phenotypic expression of several life-history traits (Alford & Harris 1988; Boes & Benard 2013; Moore & Martin 2019; Warne et al. 2013; Yagi & Green 2018). Therefore, conditions in the breeding ponds may deeply affect population dynamics: at local scales through effects on adult phenotypes and fitness and at regional scales through effects on dispersal ability (e.g., Beck & Congdon 2000; Beckerman et al. 2002; Benard & Fordyce 2003; Benard & McCauley 2008; Relyea 2001). Consequently, exploring latent effects of larval conditions on subsequent developmental stages is a key factor to understand population dynamics (Pechenik 2006). Phenotypic plasticity in developmental rate and metamorph size of *B. variegata* were investigated by Böll (2002), however with ambiguous results. Yet, no research has been reported on potential carry-over effects from larval history to the performance of *B. variegata* metamorph phenotypes and associated consequences for the survival of terrestrial stages. As anuran metamorphs are prone to invertebrate predator attacks when leaving the larval habitat (Toledo 2005), carry-over effects may contribute to the toads' vulnerability to invertebrates, such as spiders or carabids. The questions remain, however, if the observed plasticity in metamorph traits is caused by environmental factor combinations experienced during aquatic development and if carry-over effects affect survival probability of terrestrial stages.

The fast-slow continuum of the life-history trait longevity

Environmental variability as well as trade-offs between constraints of an organism shape the evolution of life-history traits (Stearns 2000). Most trait variation among species is observed in characteristic combinations that fall on a fast-slow continuum (Ricklefs & Wikelski 2002; Stearns 1983). At the two opposite ends of this continuum, there are for one part organisms of slow life histories (i.e. slow development, longevity and low fecundity) and for the other part those of fast life histories (i.e. rapid development, short lifespan, high fecundity) (Ricklefs & Wikelski 2002; Stearns 2000). Research is required to disentangle natural geographic variation and effects caused by habitat loss or climate change on demographic life-history traits of amphibian populations (Becker et al. 2018; Sinsch 2015). Yellow-bellied toads exhibit an extraordinary wide among-population variation of adult survival rates and longevity. In fact, some populations have a maximum lifespan of more than 20 years in the field, i.e. similar to individuals in captivity, while others feature a longevity of less than 10 years (e.g., Abbühl & Durrer 1998; Bülbül et al. 2018; Cayuela et al. 2019a; Di Cerbo et al. 2011; Hantzschmann et al. 2019; Mertens 1964; Mertens 1970; Seidel 1993). This broad fast-slow continuum makes *B. variegata* an excellent model organism for the study of intraspecific differences in demographic life-history traits. Yet, the causes for this broad continuum of longevity remain, to date, largely unknown.

Aspects of population structure and dynamics

The understanding of amphibian decline and of environmental factors defining the status of species requires research on population dynamics at local and metapopulation scales (Alford & Richards 1999; Beebee & Griffiths 2005; Hecnar & McLoskey 1996). The structure and dynamics among yellow-bellied toad populations vary profoundly, probably due to differing mortality risk across habitats (Gollmann & Gollmann 2012). As a pioneer species, *B. variegata* benefits from human-induced disturbances that create heterogeneous early successional habitats (Cayuela et al. 2018). However, habitat stochasticity seems to be a major factor linking dispersal and life-history traits, i.e. fecundity and lifespan, in yellow-bellied toads and thus affects population structure and dynamics (Cayuela et al. 2018; Cayuela et al. 2016c). Although a profound knowledge of local population dynamics is required to assess vulnerability to threats and to implement informed conservation management (Denoël & Lehmann 2006; Lambert et al. 2016; Muths et al. 2011), the current structure and dynamics

of one of the presumably largest remnant *B. variegata* populations in Rhineland-Palatinate is still unexplored.

Conservation genetics

In most species, geographic variation in morphology and gene frequency depends on genetic differentiation of local populations through mutation, genetic drift and natural selection on one side and genetic homogeneity through opposing gene flow on the other (Slatkin 1987). A consequence of habitat fragmentation is isolation of amphibian populations, especially of less mobile species, which affects genetic variation (Andersen et al. 2004; Ficetola & De Bernardi 2004). As reduced genetic variation is considered to decrease fitness and adaptability in amphibian populations, it may increase the vulnerability to threats and thus contribute to the worldwide decline (Allentoft & O'Brien 2010; Rowe et al. 1999). According to the central-marginal hypothesis, peripheral populations are supposed to be small and geographically isolated with lower genetic diversity and greater differentiation compared to geographically central populations (Eckert et al. 2008). To implement adequate conservation measures for key species, a profound knowledge of the underlying genetic patterns of populations is required (Balloux & Lugon-Moulin 2002; Holderegger et al. 2019). Molecular studies on yellow-bellied toads focussed on regions in France (Cayuela et al. 2017b; Vacher & Ursenbacher 2014), Italy (Cornetti 2013; Cornetti et al. 2016), Switzerland (Tournier 2017) and Germany (Guicking et al. 2017; Weihmann et al. 2009 ; Weihmann et al. 2019). Yet, parts of the northern range margin, i.e. northern Rhineland-Palatinate with its numerous populations inhabiting the Westerwald region, are still unexplored. Thus, it remains unclear, if the Westerwald populations are affected by isolation and associated negative effects on genetic diversity. Another question is whether the populations at the northern range margin are genetically less diverse than their southern counterparts.

Design and aims of the present study

In this thesis, I applied a combination of long-established and modern scientific methods to approach these open questions. Experiments to reveal carry-over effects from larval to metamorphic stage were performed using laboratory and mesocosm approaches. In amphibian ecology studies, outdoor mesocosm experiments are artificial, self-sustaining systems used to mimic natural habitats (James & Boone 2005; Wilbur 1997). Mesocosms outperform laboratory studies in environmental realism, while cause-effect relationships are

best detected under laboratory control (Semlitsch & Boone 2009). For the demographic study of yellow-bellied toads, I used a combination of indirect age assessment through non-invasive capture-mark-recapture and direct age assessment through invasive skeletochronology. These long-established methods are based on individual identification by the toads' unique ventral patterns on one side and on counting the number of hematoxylinophilic growth marks in phalange bones on the other side. Capture-mark-recapture (CMR) is a reliable method to study population demography and dynamics including survival, population growth rate, abundance and recruitment (Pradel 1996; Schmidt 2004). CMR requires accurate identification of individuals that can be achieved by invasive (e.g. toe-clipping) and non-invasive methods (e.g. using natural markings for photo-identification) (Mettouris et al. 2016). In the context of amphibian decline non-invasive marking is considered beneficial, especially when investigating rare species, as invasive methods may have negative impacts on individuals (Davis & Ovaska 2001; Sannolo et al. 2016; Shu et al. 2018). Nonetheless, invasive methods may be advantageous. Skeletochronology, for example, allows for solid estimates of adult survival and individual longevity of up to 8 years as well as the reconstruction of age structure (Sinsch 2015). Therefore, this invasive method is particularly suitable for short-term studies and was used to investigate the demographic structure of yellow-bellied toads at the local and regional scale. Informed conservation management furthermore requires the investigation of intraspecific genetic variation at local, regional and global scales, especially to understand population dynamics and evolutionary potential at the range margin of a species (Lesbarrères et al. 2014; Mimura et al. 2017). Molecular ecology provides useful tools to address these issues, including within-population processes, spatial population structure, i.e. landscape genetics, and the identification of genes for local adaptation (McCartney-Melstad & Shaffer 2015). Among these tools, microsatellites are popular and versatile marker types used for ecological applications (Selkoe & Toonen 2006). Thus, I complemented this study by using 10 microsatellite markers at the local and regional scale and put these data in a continental context.

In this thesis, life-history traits and genetics of yellow-bellied toads are explored to identify the threats *B. variegata* is facing at the range margin and to develop adequate conservation measures. The study is performed on different geographical scales. The local scale is defined by the range of members of a (single) population (i.e. the former military training area Schmidtenhöhe, see chapters 2-5). The regional scale represents a (former)

metapopulation system (i.e. the Westerwald region, see chapters 3-5). The continental scale is defined by the geographical range of (all) *B. variegata* populations (data supplemented with previously published information, see chapters 3, 5).

To test predictions of life-history theory, I focussed on several developmental stages, from the early larval period to metamorphosis and adulthood. Furthermore, I addressed developmental, demographic and genetic issues of *Bombina variegata* as a model organism. In order to fill current gaps in knowledge, this study aims at answering the following research questions:

(1) Are there interactions among the conditions experienced in the aquatic habitat during larval development and the variation of metamorph features and fitness, suggesting carry-over effects from larval to terrestrial stages in *B. variegata* (chapter I)?

(2) Does plasticity of metamorph traits affect the vulnerability of juvenile yellow-bellied toads to predators (chapter II)?

(3) What are the intrinsic and extrinsic drivers of interspecific and interannual variation of the slow-fast continuum of longevity in *B. variegata* (chapter III)?

(4) How do interannual recruitment, dispersal ability and longevity affect population dynamics and which inferences can be drawn for population persistence, connectivity and resilience of yellow-bellied toads (chapter IV)?

(5) Are there effects of habitat fragmentation on isolation and gene drift in *B. variegata* populations and differences in genetic variation among central and marginal populations (chapter V)?

The thesis is based on five studies, each presented in an individual chapter containing abstract, introduction, material and methods including a thorough description of the study area, results and a discussion, respectively. The resulting manuscripts are either submitted, accepted or already published in peer-reviewed journals.

Chapter I

The larval stage: Carry-over effects of larval environment

This article is accepted by the Herpetological Journal and will be published as:

Ulrich Sinsch[1], Fabienne Leus[1], Marlene Sonntag[1] & Alena Marcella Hantzschmann[1] (2020): Carry-over effects of the larval environment on the post-metamorphic performance of *Bombina variegata* (Amphibia, Anura). Herpetological Journal 30: 125-133

[1]Institute for Integrated Natural Sciences, Department of Biology, University of Koblenz-Landau, Universitätsstr. 1, D-56070 Koblenz, Germany

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https://www.thebhs.org/publications/the-herpetological-journal

Abstract. Metamorphs of the yellow-bellied toad *Bombina variegata* vary widely in size at metamorphosis in the field. We performed a replicated outdoor mesocosm study to simulate the environmental factor combinations in permanent and ephemeral breeding sites and to quantify their effects on tadpole development (duration of the larval period, metamorph size and body condition). Looking for potential carry-over effects of the larval environment, we quantified locomotor performance of all metamorph phenotypes originating from the mesocosms immediately after metamorphosis under controlled conditions. In contrast to the prediction of life-history theory, tadpoles were unable to adjust developmental rate to water availability, but metamorphs originating from the ephemeral pond treatment were smaller and had a lower body condition than those from the permanent pond treatment. Size-dependent carry-over effects included the length of the first jump following tactile stimulation, burst performance (total length of spontaneous jumps) and endurance (total distance covered in 10 forced jumps). A size-independent effect of larval environment was the prolonged locomotor effort to escape (5.7 consecutive jumps following initial stimulus) of metamorphs from the ephemeral pond treatment compared to same-sized ones (3.7 jumps) from the permanent pond treatment. Thus, we demonstrate that carry-over effects of larval environment on metamorph phenotype and behaviour cause a considerable variation in fitness in the early terrestrial stage of *B. variegata*. Informed conservation management of endangered populations in the northern range should therefore include the provision of small permanent breeding ponds promoting larger and fitter metamorphs.

Key words. Complex life cycles, Mesocosm, Plasticity of metamorph phenotype, Post-metamorphic jumping performance, Delayed density dependence, Delayed life history effects

Chapter II

The metamorph stage: Carabid predation

This article is published as:

Alena Marcella Schäfer[1], Francesca Schäfer[1], Thomas Wagner[1] & Ulrich Sinsch[1] (2018): Carabid predation on *Bombina variegata* metamorphs: size at and timing of metamorphosis matter. Salamandra 54 (3): 222-228

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Abstract. The potential impact of carabid predation on dispersing *Bombina variegata* metamorphs was studied in the field at the Schmidtenhöhe (Rhineland-Palatinate, Germany) and performing experimental trials in the laboratory. At least four carabid species were demonstrated to feed on metamorphs, with *Carabus violaceus* and *Pterostichus niger* mainly preying on the smallest individuals, and *Abax parallelepipedus* and *Harpalus rufipes* indiscriminately attacking smaller and larger individuals. Predation rates were mostly low (5-15% of metamorphs) despite of high prey densities (83 metamorphs per m²), but hungry beetles consumed up to 60% within five days. Plasticity in the timing of metamorphosis (about two months) and in the size at metamorphosis (11-19mm) observed in the field seemed to reflect mainly the variability in the spawning date and of aquatic environment during the tadpole development. Still, the tendency of metamorphs to be become larger on average towards the end of the metamorphosis period reduced their risk of being predated because the abundance of potential carabid predators did neither vary among habitat types crossed by dispersing metamorphs nor during the period of metamorphosis. Consequently, informed conservation management of endangered *B. variegata* populations should focus on larval habitats producing large-sized metamorphs to reduce loss of metamorphs by size-assortative carabid predation.

Chapter III

The adult stage: the fast-slow continuum of longevity

This article is published as:

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Abstract. Yellow-bellied toad populations (*Bombina variegata*) show a wide fast–slow continuum of the life-history trait longevity ranging from 5 to 23 years. We investigated populations in Germany (n=8) and Austria (n=1) to determine their position within the continuum of longevity and the potential drivers of adult survival at the local and the continental scale. Intrinsic and extrinsic factors considered were local weather, nutritional state, allocation of ingested energy to somatic growth, pathogen prevalence, and geographical clines (latitude, altitude, and longitude). Capture-mark-recapture (CMR) monitoring and direct age assessment by skeletochronology allowed for reliable estimates of longevity and adult survival. Raw and corrected recapture rates as well as a probabilistic estimate of the lifespan of the eldest 1% adults of a cohort (CMR data) were used as surrogates for adult survival and thus longevity in a population. Additionally, survival rates were calculated from static life tables based on the age structure (skeletochronological data) of eight populations. Populations in Germany were short-lived with a maximum lifespan of annual cohorts varying from 5 to 8 years, whereas the population in Austria was long-lived with a cohort longevity of 13 to 23 years. We provide evidence that annual survival rates and longevity differ among years and between short- and long-lived populations, but there was no decrease of survival in older toads (i.e. absence of senescence). Variation of weather among years accounted for 90.7% of variance in annual survival rates of short-lived populations, whereas the sources of variation in the long-lived population remained unidentified. At the continental scale, longevity variation among *B. variegata* populations studied so far did not correspond to geographical clines or climate variation. Therefore, we propose that a population´s position within the fast-slow continuum integrates the response to local environmental stochasticity (extrinsic source of variation) and the efficiency of chemical antipredator protection determining the magnitude of longevity (intrinsic source of variation).

Key words. Fast-slow continuum, Life history, Demography, Longevity, Adult mortality, Skeletochronology, Climate, Growth pattern, Palatability

Chapter IV

The adult stage: regional population dynamics and implication for conservation management

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Zusammenfassung. Die demographische und genetische Struktur von fünf Gelbbauchunken-Populationen im nördlichen Rheinland-Pfalz wurde in dieser Fallstudie untersucht, um lokale naturschutzfachliche Maßnahmen zu optimieren und überregionale Handlungsempfehlungen zu entwickeln. Dabei lag der Fokus in den untersuchten Populationen auf (1) der jährlichen Rekrutierung von Jungtieren (als Maß für die Persistenz der Population), (2) dem Ausbreitungspotential (als Maß für die Vernetzung benachbarter Populationen) und (3) der Langlebigkeit Adulter (als Maß für Resilienz, wechselnden Reproduktionserfolg zu überdauern). Die von 2016 - 2018 auf einem ehemaligen militärischen Übungsgelände bei Koblenz durchgeführte Fang-Markierung-Wiederfang-Studie (CMR) sowie eine Mikrosatelliten-Analyse auf genetische Substrukturierung deuten auf eine Fragmentierung

sowie niedriges Ausbreitungspotential der Population hin. Eine skelettochronologische Altersbestimmung weist in Kombination mit den Ergebnissen der CMR-Analyse eine niedrige Lebenserwartung dieser potenziell langlebigen Art im nördlichen Rheinland-Pfalz nach. Wir empfehlen daher zum Erhalt isolierter und kurzlebiger Populationen: (1) neben temporären auch permanente, sowie neben besonnten auch teils beschattete Gewässer als Laichhabitate anzubieten, (2) Trittsteingewässer zur Vernetzung fragmentierter Populationen anzulegen und (3) Artenschutzmaßnahmen in regelmäßigen, kurzen Abständen durchzuführen.

Stichworte. Amphibien, Anuren, Naturschutzmanagement, *Bombina variegata*, Skelettochronologie, Langlebigkeit, Mikrosatelliten-Marker, Populationsgenetik, Habitatfragmentierung

Abstract. The demographic and genetic structure of *Bombina variegata* populations was studied in northern Rhineland-Palatinate, Germany, to optimise local conservation measures and to develop transregional recommendations for action. This case study focussed on estimating (1) annual recruitment of metamorphs (as a measure for population persistence), (2) dispersal (as a measure for connectivity among populations) and (3) longevity (as a measure for resilience at varying reproductive success) of yellow-bellied toad populations. A capture-mark-recapture (CMR) study from 2016 to 2018 and a microsatellite analysis of genetic substructuring revealed fragmentation due to low dispersal capacity in populations inhabiting a former military training area near Koblenz. CMR and skeletochronology demonstrated that this potentially long-lived species has a very short lifespan in northern Rhineland-Palatinate. The conservation of fragmented and short-lived *B. variegata* populations requires (1) the construction of temporary and permanent ponds for reproduction, (2) stepping stone ponds for the connectivity of fragmented populations and (3) habitat management measures at regular, short intervals.

Key words. Amphibians, Anurans, Conservation management, *Bombina variegata*, Skeletochronology, Longevity, Microsatellite markers, Population genetics, Habitat fragmentation

Chapter V

The adult stage: population genetics

Alena Marcella Hantzschmann[1], Ulrich Sinsch[1], Christa Göttlicher[1] & Heike Pröhl[2]: Conservation genetics of yellow-bellied toads (*Bombina variegata*): a matter of geographical scale and connectivity among populations

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Abstract. The yellow-bellied toad *Bombina variegata* is a threatened and strictly protected amphibian species in the marginal regions of distribution. We studied the genetic structure and diversity of populations at three geographical scales to detect potential sources of threat for the persistence of populations. At the local scale, we sampled four neighbouring localities at 1 – 2.6 km distance and five additional localities at the regional scale at up to 50.1 km distance within the low mountain range of the Westerwald (200 – 480 m a.s.l., Rhineland-Palatinate, Germany). Population connectivity and genetic diversity of 182 specimens were investigated using ten microsatellite markers. At the continental scale, we completed the data obtained in the Westerwald region with data published in studies on the Hesse and Lower Saxony regions (Germany), Alsace (France), Geneva (Switzerland), and Trentino (Italy) to evaluate variation of landscape genetics among marginal and more central populations. Short-term fragmentation of populations with low dispersal capacity caused significant genetic differentiation between populations only 1.4 km distant from each other, but did not affect local genetic diversity. Long-term isolation of populations was associated with a steep increase of genetic distance at the regional scale and the local loss of genetic diversity. At the

continental scale, we identified Alsace and Trentino as regions with low genetic structuring and high allelic richness, and the marginal or strongly urbanized remaining regions as deeply structured with reduced allelic richness. Our study suggests that informed conservation management of *B. variegata* should focus on measures maintaining or improving connectivity among neighbouring populations.

Key words. Species conservation, Geographical scale, Microsatellites, Genetic diversity, Isolation by distance, Habitat fragmentation, Conservation management

Introduction

Worldwide declines of amphibians affect increasing numbers of species (IUCN 2019; Stuart et al. 2004). Besides habitat loss identified as main cause, environmental contaminants, UV-B irradiation, diseases, invasive species, exploitation and climate change contribute to the observed declines (Beebee & Griffiths 2005; Hof et al. 2011). Reduced genetic variation may further increase vulnerability of amphibian populations to decline (Allentoft & O'Brien 2010; Chen et al. 2012). Genetic diversity can vary greatly among conspecific amphibian populations in response to effective population sizes, isolation of populations and bottleneck effects (Funk et al. 2005; Monsen & Blouin 2004; Razpet et al. 2016). Therefore, molecular tools (e.g., microsatellite analyses) are commonly used to assess genetic diversity in target species and to reveal fragmentation and connectivity among populations for conversation purposes (Balloux & Lugon-Moulin 2002; Holderegger et al. 2019). For example, a continental scale study on the declining Australian frog *Litoria aurea* emphasized that connectivity among neighbouring populations is crucial to counteract genetic structuring (Burns et al. 2004).

The central-marginal hypothesis predicts that populations at the margin of the geographical range are usually small and geographically isolated, with low genetic diversity and high genetic divergence (Eckert et al. 2008; Peterman et al. 2013). The phylogeographies of the European tree frog *Hyla arborea* and the natterjack toad *Epidalea calamita* support this prediction because northernmost populations established during the postglacial recolonization are genetically less diverse than southern populations in the glacial refuge areas and more prone to local extinction (Allentoft et al. 2009; Dufresnes et al. 2013; Rowe et al. 1998; Rowe et al. 2006). The European yellow-bellied toad *Bombina variegata* shares a

similar postglacial recolonization history (Gollmann & Gollmann 2012). At continental scale, this species is currently listed "least concern"; but protected under the EU Habitats Directive 92/43/EE and considered endangered in many European countries (IUCN 2019; Kühnel et al. 2009). At its northern range limit in Germany, many yellow-bellied toad populations have gone extinct mainly due to habitat loss, while others are declining during the past decades (Gollmann & Gollmann 2012; Veith 1996a). As extant populations are often fragmented because of a low dispersal capacity and habitat fragmentation, *B. variegata* is presently considered "critically endangered" in Germany (e.g., Gollmann & Gollmann 2012; Jehle & Sinsch 2007; Kühnel et al. 2009; Schlüpmann et al. 2011; Veith 1996a).

We chose yellow-bellied toads as a model organism to explore genetic diversity and isolation by distance patterns among populations at the local, regional, and continental scale. Using microsatellite analyses, we aimed to test for the effects of habitat fragmentation on isolation and gene drift in central and marginal populations and to evaluate implications for conservation. We hypothesise that connectivity among marginal populations is lower than among central populations, resulting in lower local genetic diversity and stronger regional genetic structuring.

(1)   At the local scale, we used a system of four neighbouring populations (1.0 - 2.5 km distant from each other; Schmidtenhöhe, Rhineland-Palatinate, Germany; Hantzschmann & Sinsch 2019) originating from a single panmictic population and separated by dispersal barriers for about 30 - 40 years. We quantified the effects of short-term habitat fragmentation on genetic structure. We predict that the interruption of gene flow caused a detectable genetic structuring of the populations; but did not yet affect the level of genetic diversity.

(2)   At the regional scale, we complemented the small-scale population system with another five populations (4 - 50 km distant from each other; Westerwald, Rhineland-Palatinate, Germany) among which exchange of individuals has been interrupted for at least a century. We quantified the long-term impact of dispersal barriers on the genetic diversity. We predict that the actual genetic structuring of Westerwald populations reflects the common origin from an initially large-scale meta-population with interacting local populations and gene drift in the isolated extant populations. We expect a detectable decrease of local genetic diversity and a strong genetic structuring within this marginal region.

(3)   At the continental scale, we performed a meta-analysis on genetic diversity in marginal and central populations. We complemented our data on the Westerwald region with

published data on regions in France (Vacher & Ursenbacher 2014), Italy (Cornetti 2013; Cornetti et al. 2016), Switzerland (Tournier 2017), and Germany (Hesse: Guicking et al. 2017; Lower Saxony: Weihmann et al. 2009; Weihmann et al. 2019). We predict that local and regional genetic diversity increases from north to south and that the isolation by distance pattern is more pronounced in the marginal regions (e.g., Germany) than in the more central regions (e.g., Italy).

Material and Methods

Study area and sampling procedure

We sampled yellow-bellied toads *Bombina variegata* from nine populations in Rhineland-Palatinate, Germany (**Fig. 1, Tab. 1**). At the local scale (approx. 2.5 km² survey area; **Fig. 1A**), we sampled four neighbouring populations (1-4, **Tab. 1**) located at the former military training area Schmidtenhöhe near Koblenz (Hantzschmann et al. 2019; Hantzschmann & Sinsch 2019). About 40 years ago, these populations formed a single panmictic population considered as the largest *B. variegata* population in Rhineland-Palatinate (Veith 1996a). At the regional scale (approx. 1,000 km² survey area; **Fig. 1B**), we sampled another five populations (5-9, **Tab. 1**) inhabiting clay and loamy sand pits in the Westerwald region of Rhineland-Palatinate. Geographical distances between localities are given as line-of-sight distance between the centres of breeding pond groups. The pairwise geographical distances range from 1.0 to 50.1 km (**Tab. 5**).

We hand-captured and toe-clipped 16-30 individuals per population, obtaining 182 samples for microsatellite-analyses (**Tab. 1**). We sampled populations 1-4 in May and July 2017, populations 5-8 in July 2018 and population 9 in June 2019. We avoided replicate sampling by checking individuals for clipped toes and comparing the unique ventral pattern with the photographs of previously collected toads. For the continental-scale meta-analysis (**Fig. 1C**), we retrieved published data throughout the western range of *B. variegata*. Populations included are at the northern range margin in Germany (Lower Saxony: Weihmann et al. 2009; Weihmann et al. 2019; Hesse: Guicking et al. 2017), in Switzerland (Tournier 2017), in France (Vacher & Ursenbacher 2014) and in Italy representing the southernmost region in this dataset (Cornetti 2013).
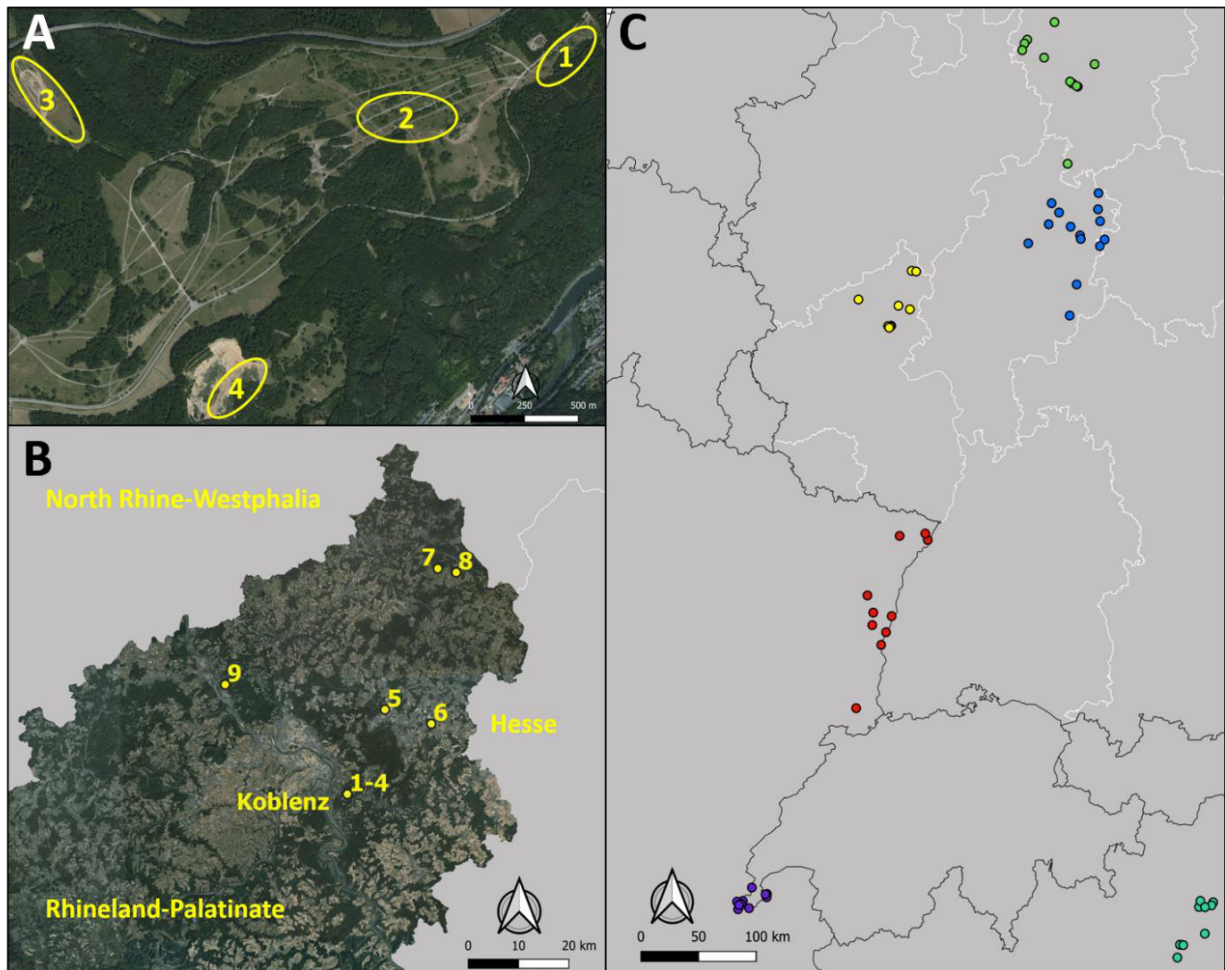
Figure 1: Geographical distribution of study sites. Local scale (A): Former military training area Schmidtenhöhe. Regional scale (B): Northern Rhineland-Palatinate (populations 1 - 4: Schmidtenhöhe; 5: Mogendorf; 6: Ruppach-Goldhausen; 7: Elkenroth; 8: Galgenkopf; 9: Bad Hönningen). Continental scale (C): populations in Lower Saxony (green); Hesse (blue); Rhineland-Palatinate (yellow); Alsace (red); Geneva (purple); Trentino (turquoise). See Table 1 for coordinates. Maps created using data by the Naturschutzverwaltung Rheinland-Pfalz; Geobasisdata: Kataster- und Vermessungsverwaltung Rheinland-Pfalz and GADM online online platform (https://gadm.org/data.html).

Table 1: Sampling localities in northern Rhineland-Palatinate with corresponding population code, land use at each site and number of individuals sampled. Local scale (Schmidtenhöhe) is indicated by light grey; regional scale white.

Sampling localities	Pop. code	Latitude [°N]	Longitude [°E]	Altitude [m asl]	Indiv. [n]	Actual land use
Schmidtenhöhe	1	50.348920	7.678354	339	20	Succession
Schmidtenhöhe	2	50.345374	7.668822	333	25	Pasture
Schmidtenhöhe	3	50.346560	7.644067	275	25	Pioneer area
Schmidtenhöhe	4	50.335656	7.655866	301	30	Clay pit (extensive)
Mogendorf	5	50.487802	7.758493	308	16	Clay pit (intensive)
Ruppach	6	50.462444	7.885331	276	16	Clay pit (intensive)
Elkenroth	7	50.727676	7.906109	480	16	Former loamy sand pit
Galgenkopf	8	50.723098	7.954793	451	18	Former loamy sand pit
Bad Hönningen	9	50.530513	7.314777	200	16	Clay pit (extensive)

DNA extraction and genotyping

We genotyped the toads using 10 colour-labelled microsatellite markers originally developed for *Bombina bombina* (Hauswaldt et al. 2007; Stuckas & Tiedemann 2006). DNA was extracted from phalange tissue (toe clips) using the blood and tissue DNA extraction kit (Qiagen). DNA was then amplified in a locus-specific polymerase chain reaction (PCR) on a MultiGene OptiMax (Labnet International) in 10 µl volumes containing 4.55–5.55 µl dH2O, 1 µl Buffer (Bioline), 5 pmol (1.0 µl) Primer, 0.3–0.4 µl My-Taq-Polymerase (Bioline) and 1–2 µl undiluted DNA. PCR conditions followed the protocol of Weihmann et al. (2009) using reduced elongation (3 min) and adapted annealing temperatures. For the B13 locus, we used a different procedure: 10 µl volume containing 3.45–6.45 µl dH2O, 2 µl HF-Buffer (Thermo Fisher), 0.2 µl dNTPs, 0.5 µl MgCl2, 0.1 µl Phusion High Fidelity-Polymerase (Thermo Fisher), 5 pmol (1.0 µl) per primer and 1.0– .0 µl undiluted DNA denaturated at 98 °C, followed by 35 cycles at 98 °C (10 s), 41 °C (30 s), 72 °C (30 s) and a final elongation at 72 °C (8 min). Fragment lengths were analysed by Eurofins Genomics, Germany, at an ABI 3130 XL sequencing machine, allele sizes were determined using the software GeneMapper 5.

Population genetic analyses

We checked the resulting fragment lengths using the software Microchecker 2.2.3 for null alleles (Van Oosterhout et al. 2004). We calculated allele number and private alleles with corresponding frequencies and genetic isolation by geographic distance (IBD) using a Mantel test in GeneAlEx (Peakall & Smouse 2012). Allelic richness (AR), deviations from Hardy-Weinberg equilibrium using Fisher's exact test, and inbreeding coefficients (F_{IS}) with corresponding confidence intervals were calculated using the package diveRsity 1.9.9 in R (Keenan et al. 2013). Linkage disequilibrium between loci (10000 permutations), an analysis of molecular variance (AMOVA); observed (H_o) and expected (H_e) heterozygosity with corresponding standard deviation, and pairwise genetic difference between populations (weighed F_{ST} -statistics according to Weir & Cockerham 1984; Michalakis & Excoffier 1996) were calculated using the software Arlequin 3.5.2.2 (Excoffier & Lischer 2010). We transformed the raw F_{ST} values using the formula $F_{ST}/(1-F_{ST})$ for the IBD analysis (Vacher & Ursenbacher 2014). Genetic bottlenecks were detected using three different methods: Wilcoxon's sign rank test for (1) stepwise mutation model (SMM), (2) two-phase model (TPM), and (3) mode shift test using the software bottleneck 1.2.02 (Piry et al. 1999). We performed a Discriminant Analysis of Principal Components (DAPC) to describe diversity between populations using the package adegenet in R (Jombart 2008). The most probable number of genetic clusters was estimated in Structure 2.3 (Pritchard et al. 2009) using the admixture model without prior information on sample population. The evaluated number of clusters (k) was set from 1 to 10 with 20 runs per k after a burn-in period of 100000 followed by 500000 iterations. We used the Evanno method (Δk; Evanno et al. 2005) in Structure Harvester (Earl & Vonholdt 2012) to evaluate the most appropriate number of genetic clusters. Corresponding charts were obtained using CLUMPAK (Kopelman et al. 2015).

Statistical analyses for continental scale comparisons

We used an analysis of variance (ANOVA) to compare the data sets on the six regions studied with respect to AR and H_e, i.e. genetic diversity. If ANOVA detected significant differences among the regions, we applied a multiple regression analysis to test for the impact of latitude, longitude and altitude on the target variables. Within each region, the isolation by distance pattern was modelled by calculating the linear relationship between geographical distance and transformed F_{ST}-value ($F_{ST}/[1-F_{ST}]$). Slopes of regression lines as a measure of intensity of

isolation among regional populations were compared using the 95 % confidence intervals. Statistical procedures were performed using the program package Statgraphics Centurion version 18.1.01. The significance level was set at alpha = 0.05.

Results

Eight of the ten loci studied were polymorphic in the Westerwald region (**Tab. 2**). The MicroChecker analysis did not provide evidence for null alleles or large allelic dropout in any of the populations. Consequently, all sampled individuals were included in the analyses. We detected 40 alleles including eight private alleles (**Tab. 2, Tab. 3**). The average number of alleles per locus was 4.2 (range: 1 - 8; **Tab. 2**). Linkage disequilibrium was found in six out of nine populations (sites 1, 5 - 9), but there was no evidence for a general linkage disequilibrium between particular loci. We did not detect deviations from Hardy-Weinberg equilibrium (Fisher's exact test; P > 0.05) or evidence for significant inbreeding in any of the investigated populations (**Tab. 3**).

Table 2: Continental scale: Fragment lengths of microsatellite loci in *B. variegata* populations of five European regions. Data are given as number of individuals (n), number of alleles (A), size range of alleles, and observed heterozygosity (H_o) per locus, if available.

Country	Germany (Rhineland-Palatinate)			Germany (Hesse)			Germany (Lower Saxony)		Italy (Trentino)		France (Alsace)		
n	182			281			150		200		290		
Reference	This study			Guicking et al. (2017)			Weihmann et al. (2019)		Cornetti (2013)		Vacher and Ursenbacher (2014)		
Locus	A	Size range	H_o	A	Size range	H_o	A	Size range	A	Size range	A	Size range	H_o
F22	2	138-140	0.05	1	143	-	6	137-169	2	142-148	5	134-146	0.77
B14	3	163-169	0.48	6	160-172	0.4	6	138-200	5	164-172	-	-	-
B13	3	112-120	0.19	1	115	-	13	95-161	3	114-134	5	114-124	0.24
5F	7	112-148	0.51	9	115-163	0.54	10	91-163	3	116-148	11	110-166	0.76
9H	3	148-156	0.17	4	151-163	0.32	9	119-203	6	156-176	11	131-183	0.57
F2	1	485	-	1	468	-	10	270-378	-	-	-	-	-
1A	1	320	-	1	323	-	8	323-383	2	322-326	-	-	-
8A	6	313-333	0.60	10	283-339	0.53	11	291-363	6	291-331	9	288-332	0.57
10F	8	192-228	0.57	5	209-225	0.58	7	193-229	7	206-230	11	197-233	0.59
12F	6	209-233	0.47	4	143-163	0.37	6	213-233	8	219-247	-	-	-

Table 3: Local and regional scale: Genetic features of the populations in northern Rhineland-Palatinate. Abbreviations: allele number (A), number and frequency of private alleles (PA), expected (H_e) and observed (H_o) heterozygosity with corresponding standard deviation (SD), allelic richness (AR), inbreeding coefficient (F_{IS}) with corresponding 95 % confidence interval (CI). Significance levels (P] of tests for genetic bottlenecks are given as well. Abbreviations: one-tailed Wilcoxon's test TPM (BN W_{TPM}), one-tailed Wilcoxon's test SMM (BN W_{SMM}), mode shift test (BN MS).

Pop. code	A [n]	PA [n]	PA [freq]	H_e (±SD)	H_o (±SD)	AR	F_{IS} (CI)	BN W_{TPM}	BN W_{SMM}	BN MS
1	21	1	0.03	0.45 (±0.21)	0.45 (±0.2)	2.51	-0.01 (-0.16-0.14)	0.23	0.47	-
2	25	0	0	0.44 (±0.23)	0.46 (±0.26)	2.68	-0.06 (-0.17-0.04)	0.77	0.95	-
3	17	0	0	0.43 (±0.23)	0.45 (±0.26)	2.11	-0.09 (-0.24-0.08)	0.04	0.05	shifted
4	20	0	0	0.45 (±0.11)	0.45 (±0.12)	2.25	-0.01 (-0.13-0.11)	0.08	0.72	-
5	22	0	0	0.41 (±0.18)	0.42 (±0.22)	2.61	-0.06 (-0.19-0.07)	0.32	0.73	-
6	25	3	0.16	0.48 (±0.22)	0.46 (±0.21)	2.94	0.01 (-0.13-0.17)	0.04	0.27	-
7	20	0	0	0.37 (±0.21)	0.37 (±0.24)	2.38	-0.03 (-0.20-0.14)	0.27	0.58	-
8	21	3	0.16	0.48 (±0.23)	0.51 (±0.22)	2.43	-0.09 (-0.24-0.05)	0.08	0.34	-
9	17	1	0.19	0.45 (±0.15)	0.47 (±0.12)	2.11	-0.08 (-0.21-0.05)	0.08	0.22	shifted

Genetic diversity

Local scale (Schmidtenhöhe)

The expected heterozygosity H_e of the Schmidtenhöhe populations was similar at the four localities (range: 0.43 - 0.45; **Tab. 3**). Population 3 had the lowest number of alleles and lowest AR and showed evidence for a bottleneck event (one-tailed Wilcoxon's test for two-phase model: $P < 0.05$, positive mode shift test; **Tab. 3**). Population 2 had the highest number of alleles and the highest AR (**Tab. 3**). Private alleles were found in low frequency in population 1 (**Tab. 3**).

Regional scale (Westerwald)

H_e of nine Westerwald populations varied in a broader range than that at the local scale (range: 0.37 - 0.48; **Tab. 3**). Population 7 had the lowest H_e, populations 6 and 8 the highest. Population 6 showed the highest AR (**Tab. 3**). Private alleles were found in populations 6, 8, and 9 in high frequencies (range: 0.16 - 0.19; **Tab. 3**). As only one of the three bottleneck tests was significant in populations 6 and 9, we did not find unambiguous evidence for a recent bottleneck event in the investigated populations.

Continental scale (Europe)

Allelic richness of *B. variegata* populations differed significantly among populations in Germany, France and Italy (ANOVA: $F_{4,52} = 13.99$, $P < 0.0001$; **Tab. 4**). Specifically, AR was significantly lower in the three marginal regions in Germany than that in the more central regions of France and Italy (Multiple group comparison: $P < 0.05$). Variation of latitude, longitude, and altitude of localities studied accounted for 41.8 % of variance in AR (Multiple regression model: $AR = 15.04–0.22*Latitude-0.11*Longitude–0.00069*Altitude$; $F_{3,52} = 13.48$, $P < 0.0001$). Specifically, AR decreased significantly from south to north and from east to west. In contrast, H_e was similar in five out of six regions studied (ANOVA: $F_{5,64} = 3.32$, $P = 0.0104$; **Tab. 4**). The only significant deviation was detected between the Geneva and Alsace regions (Multiple group comparison: $P < 0.05$). Latitude, longitude, and altitude of localities did not account for a significant amount of variance in H_e (Multiple regression analysis: $F_{3,64} = 1.62$, $P = 0.1937$).

Table 4: Continental scale: Average allelic richness (AR), expected heterozygosity (H_e) and corresponding 95 % confidence intervals. Hyphenated letters refer to groups differing at P < 0.05. n.a. means unspecified.

Geographic region	AR 95 % CI	H_e 95 % CI	Reference
Rhineland-Palatinate, Germany	2.45 [a] 2.27-2.63	0.44 [a,b] 0.42-0.46	This study
Lower Saxony, Germany	2.41 [a] 2.15-2.69	0.43 [a,b] 0.38-0.48	Weihmann et al. (2019)
Northern Hesse, Germany	2.53 [a] 2.36-2.71	0.48 [a,b] 0.44-0.51	Guicking et al. (2017)
Alsace, France	3.59 [b] 3.18-3.99	0.51 [b] 0.45-0.56	Vacher and Ursenbacher (2014)
Trentino, Alto Adige, Italy	3.14 [b] 2.86-3.43	0.47 [a,b] 0.43-0.51	Cornetti (2013)
Geneva, Switzerland	n.a.	0.41 [a] 0.38-0.44	Tournier (2017)

Landscape genetics

Local scale (Schmidtenhöhe)

At the local scale, 96 % of genetic variation was explained by variation within populations, only 4 % were attributable to variation among populations (AMOVA with weighted F_{ST} statistics over all loci: < 0.001). Populations that were at least 1.4 km distant from each other showed a corresponding genetic differentiation at the Schmidtenhöhe (AMOVA with weighted F_{ST} statistics over all loci: P < 0.05; **Tab. 5**). Populations 1 and 2 at about 1 km distance did not show a significant genetic differentiation.

Table 5: Local and regional scale: Matrix of pairwise genetic (F_{ST}- values; lower triangle) and geographic distance (km; upper triangle) among the Westerwald populations. Local scale (Schmidtenhöhe) is shown in grey. Significant genetic distances (P < 0.05) are given in bold.

Pop. Code	1	2	3	4	5	6	7	8	9
1	-	1.0	2.6	2.3	16.3	19.3	45.1	46.0	32.7
2	0.01	-	1.6	1.4	17.0	20.3	45.8	46.7	32.2
3	**0.08**	**0.05**	-	1.6	17.6	21.5	46.3	47.4	30.9
4	**0.03**	**0.04**	**0.04**	-	18.4	21.6	47.2	48.1	32.4
5	**0.03**	**0.07**	**0.08**	**0.04**	-	9.5	28.8	29.8	31.6
6	**0.16**	**0.19**	**0.28**	**0.2**	**0.14**	-	29.6	29.5	41.1
7	**0.09**	**0.15**	**0.23**	**0.16**	**0.11**	**0.13**	-	3.6	47.1
8	**0.19**	**0.2**	**0.28**	**0.27**	**0.24**	**0.23**	**0.14**	-	50.1
9	**0.2**	**0.25**	**0.29**	**0.28**	**0.15**	**0.27**	**0.21**	**0.35**	-

<div align="center">Regional scale (Westerwald)</div>

At the regional scale, 83.9 % of genetic variation was explained by variation within populations, whereas 16.1 % of the variation was attributable to variation among populations (AMOVA with weighted F_{ST} statistics over all loci: P < 0.001). We found significant genetic differentiation between population pairs covering a distance range of 1.4 – 50.1 km (**Tab. 5**) with a significant global F_{ST} value of 0.16 (AMOVA with weighted F_{ST} statistics over all loci: P < 0.001; **Tab. 6**). The analysis of IBD showed a significant positive correlation between genetic and geographic distance (Mantel test: P < 0.05, R^2 = 0.45; IBD = 0.06+0.0055*Geographical distance [km]; **Fig. 2**). The most likely population structure using the Evanno method suggested three different genetic cluster of *B. variegata* in northern Rhineland-Palatinate (**Fig. 3A**). Populations 1–4 are assigned to cluster 1, populations 6 and 9 to cluster 2 and populations 7 and 8 to cluster 3 (**Fig. 3B**). Population 5 has an intermediate position between clusters 1 and 2 (**Fig. 3B**). In contrast, the Discriminant Analysis of Principal Components yielded a differentiated pattern of the genetic structure of *B. variegata* with population 9 being the most distinct population (**Fig. 4**).

Table 6: Continental scale: Average genetic differentiation of *B. variegata* populations inhabiting six European regions. IBD is modelled by linear regression analyses. Slopes and intercepts of the regression models are given as least square means and corresponding 95 % confidence intervals. Hyphenated letters refer to groups differing at P < 0.05; * means significantly distinct from zero at P < 0.05; ns means not significantly distinct from zero (P > 0.05).

Geographic region	Global F_{ST}	R² [%]	Slope 95 % CI	Intercept 95 % CI	Reference
Rhineland-Palatinate, Germany	0.16	41.7 % [*]	0.00531 [a] 0.00310-0.00750 [*]	0.0687 [a] 0.0007-0.1367 [*]	This study
Lower Saxony, Germany	0.19	10.9 % [*]	0.00172 [a,b] 0.00034-0.00310 [*]	0.1709 [a] 0.1050-0.2368 [*]	Weihmann et al. (2019)
Northern Hesse, Germany	0.21	8.8 % [*]	0.00220 [a,b] 0.00071-0.00382 [*]	0.2207 [a] -0.1525-0.2889 [ns]	Guicking et al. (2017)
Alsace, France	0.13	0.2 % [ns]	0.00008 [b] -0.00198-0.00214 [ns]	0.1384 [a] 0.0684-0.2784 [*]	Vacher and Ursenbacher (2014)
Trentino, Alto Adige, Italy	0.11	0.1 % [ns]	0.00018 [b] -0.00093-0.00129 [ns]	0.1637 [a] 0.0877-0.2397 [*]	Cornetti (2013)
Geneva, Switzerland	0.12	1.1 % [ns]	0.00096 [a,b] -0.00124-0.00223 [ns]	0.1306 [a] 0.0879-0.1736 [*]	Tournier (2017)
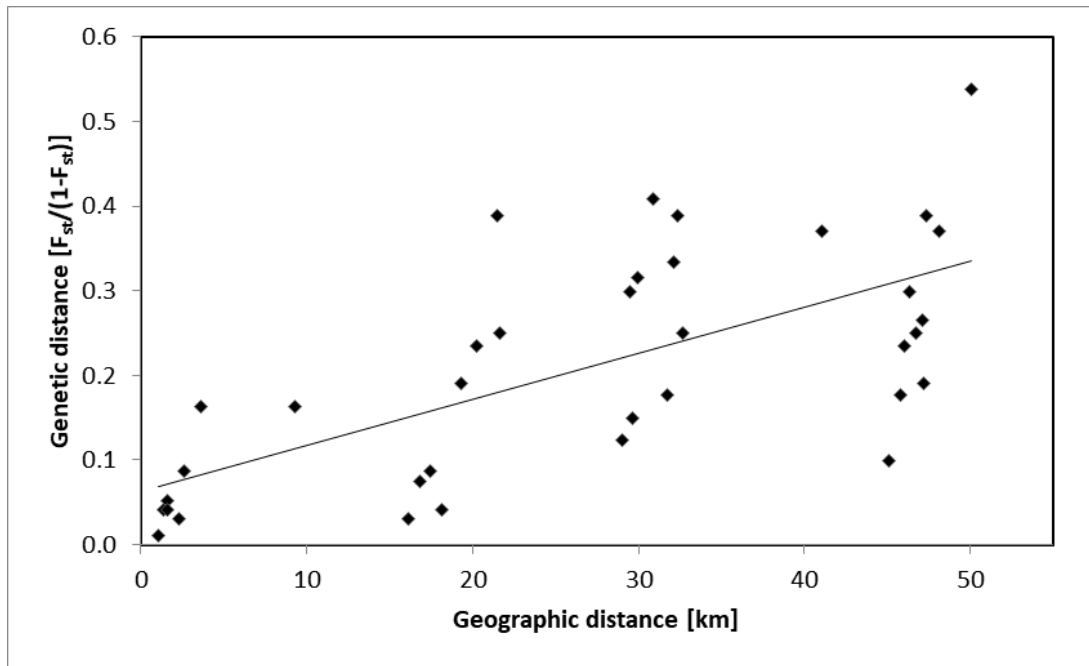
Fig. 2. Isolation by distance: regression of genetic distance ($F_{ST}/(1-F_{ST})$) on geographic distance in the Westerwald region.
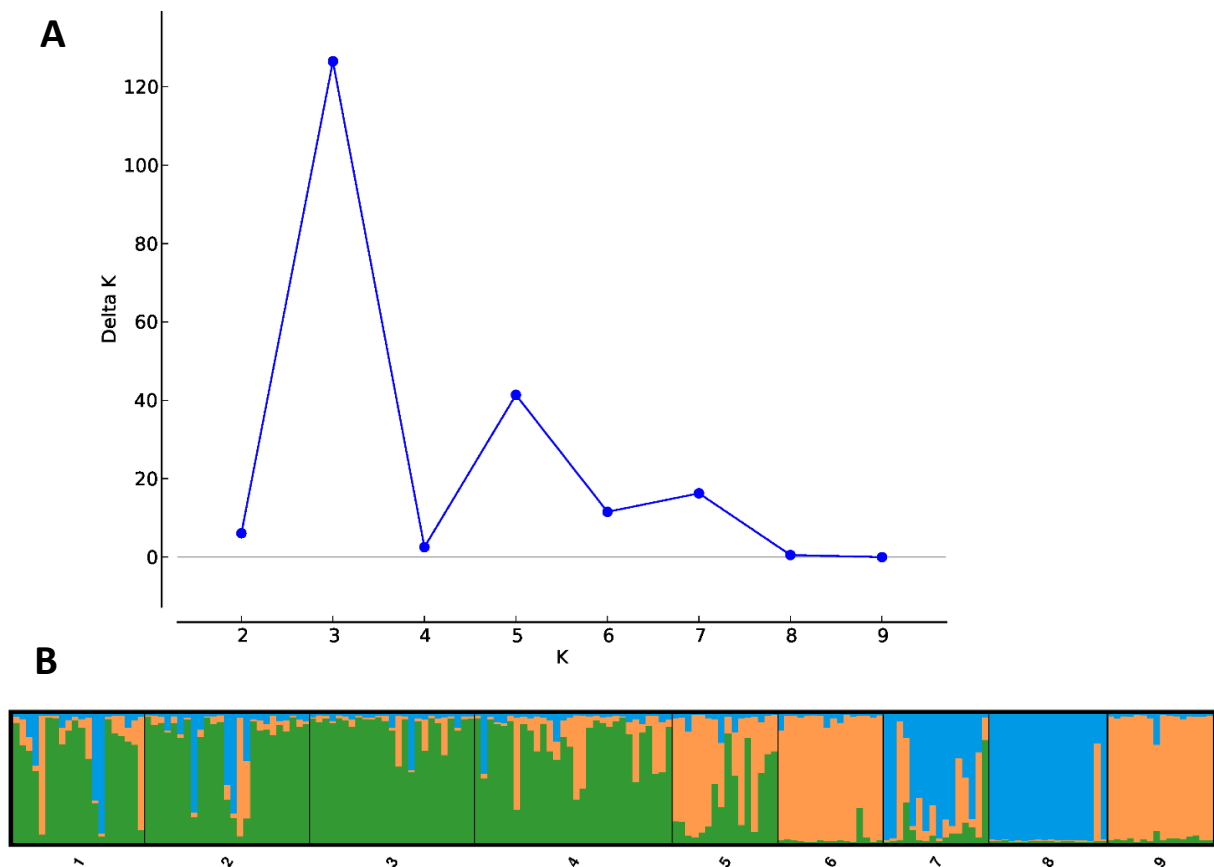

Fig. 3. A Delta K plot representing the most probable number (K = 3) of genetic groups in the Westerwald region using the Evanno method. **B.** Results of the structure analysis of populations in the Westerwald region representing three (K = 3) genetic clusters.
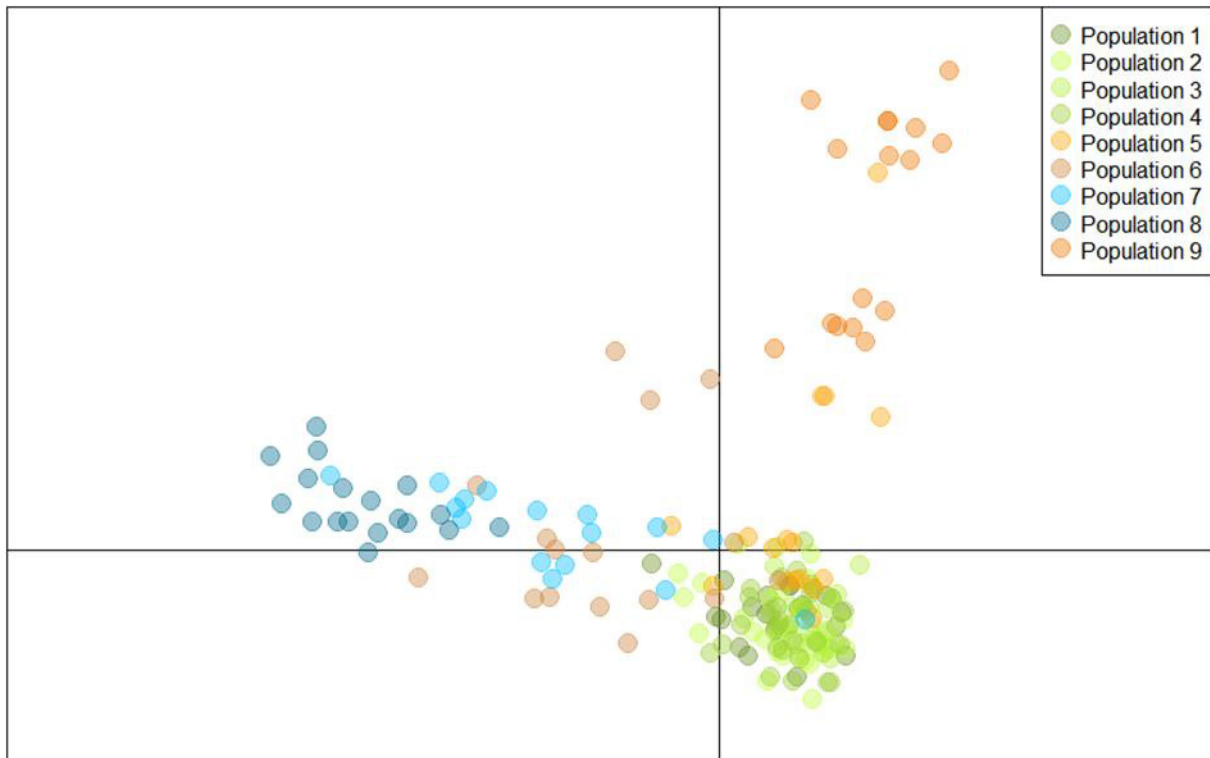
Fig. 4. Results of the DAPC (discriminant analysis of principal components) on populations in the Westerwald region.

Continental scale (Europe)

The genetic differentiation between pairs of populations within the six regions studied differed considerably between the southern and the northern regions (**Tab. 6, Fig. 5**). Isolation by distances up to 150 km was low in Trentino (Italy) and Alsace (France), whereas it was up to four times larger in a distance range of 20 - 70 km in the German and Swiss regions. There were exceptions from the rule in all regions, but the greatest genetic differentiation between population pairs was detected in the neighbouring regions Hesse and Rhineland-Palatinate at the northern range limit of *B. variegata*. Despite of the regional scatter of pairwise genetic differentiation, IBD regression models differed significantly with respect to slopes between Rhineland-Palatinate on one side and Alsace and Trentino on the other (**Tab. 6**).
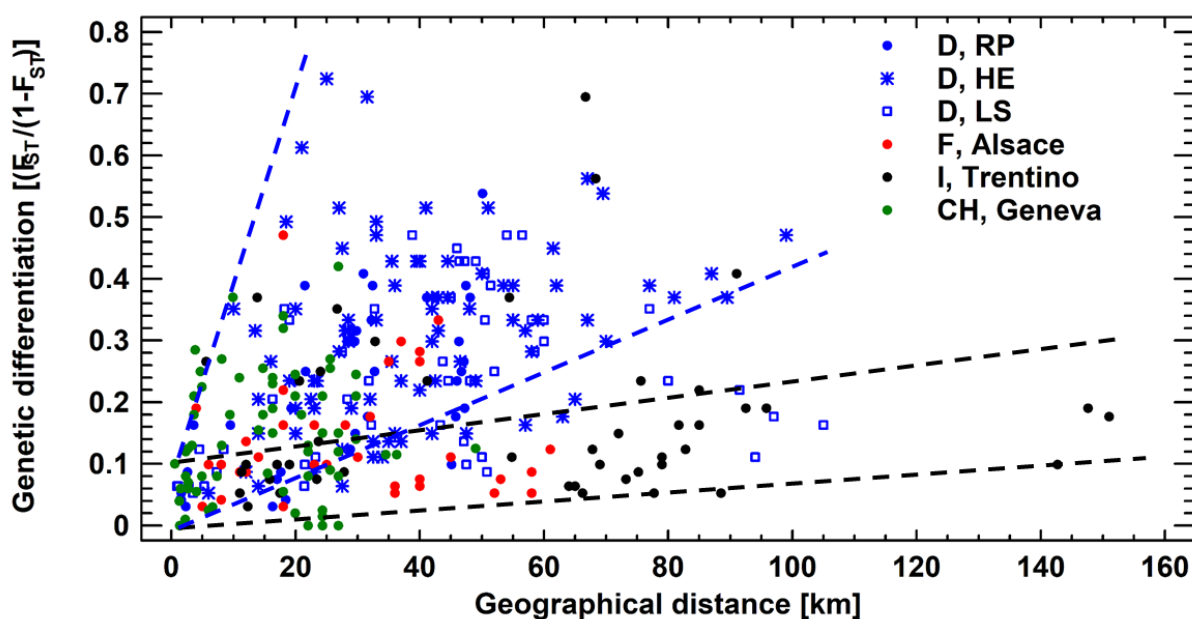
Fig. 5. Plot of geographical and genetic distances in population pairs of *B. variegata* in six European regions. Broken lines indicate the range of variation of populations with restricted (blue) and strong gene flow (black). Abbreviations: D: RP = Westerwald, Rhineland-Palatinate, Germany (this study); D: LS = Lower Saxony, Germany (Weihmann et al. 2019); D: HE = northern Hesse, Germany (Guicking et al. 2017); F: Alsace = Alsace, France (Vacher and Ursenbacher 2014); I: Trentino = Trentino, Alto Adige, Italy (Cornetti 2013); CH: Geneva = Geneva, Switzerland (Tournier 2017).

Discussion

The landscape genetics of anuran species and the subsequent genetic structuring of populations in their geographical range are shaped by gene flow, i.e. connectivity, among neighbouring populations (Frei et al. 2016; Waraniak et al. 2019; Zancolli et al. 2014). The ability of individuals to reach a neighbouring conspecific population, i.e. movement capacity, depends on density-dependent motivation to disperse, landscape resistance, and life expectancy (Cayuela et al. 2016c; Hantzschmann et al. 2019; Sinsch 2014; Stevens et al. 2006). Capture-mark-recapture (CMR) studies suggest that the annual migratory range of *B. variegata* is 20 - 732 m (Abbühl & Durrer 1996; Beshkov & Jameson 1980; Hantzschmann & Sinsch 2019; Hartel 2008; Jacob et al. 2009; Jordan 2012), whereas lifetime dispersal distance in long-lived populations may amount 1.2 - 4.5 km (Gollmann & Gollmann 2005; Jehle & Sinsch 2007; Plytycz & Bigaj 1984). Thus, exchange of individuals between neighbouring *B. variegata* populations seems to be limited to less than 5 km. As Hantzschmann et al. (2019) showed that *B. variegata* populations inhabiting the northern range margin in the Westerwald region are

short-lived, migration is probably limited to much shorter distances than in long-lived populations. We discuss the observed landscape genetics of yellow-bellied toads in the context of local, regional and continental scale and evaluate the predictions on genetic structuring in view of the short-term and long-term isolation of populations in the Westerwald region.

Local scale

As predicted, the small-scale population system studied at the Schmidtenhöhe mirroring a 30 to 40-years history of habitat fragmentation was already genetically structured indicating the impact of gene drift on small-sized local populations. This is in agreement with a similar genetic structuring in populations inhabiting the Geneva region in which structuring was attributed to habitat fragmentation by urbanisation (Tournier 2017). Dry open grasslands without water bodies pose a high landscape resistance for movements of *Bufo bufo*, *Rana dalmatina* and *Lissotriton vulgaris* (Jeliazkov et al. 2019). CMR surveys at the Schmidtenhöhe did not provide evidence for among-patch migrations of yellow-bellied toads suggesting that these landscape elements may constitute effective dispersal barriers for these toads as well (Hantzschmann & Sinsch 2019). As the number of individuals reaching neighbouring breeding sites decreases with increasing distance between ponds (Hartel 2008), dispersal in the Schmidtenhöhe populations seems to be limited by the absence of ponds that serve as stepping stones. Genetic diversity of local populations remained unaffected by short-term isolation as predicted suggesting that a notable loss of alleles due to genetic drift is a long-term process in this species. We conclude that in small-sized yellow-bellied toad populations random variation of local allele frequencies is a process leading to genetic structuring within a few decades.

Regional scale

Modelling genetic structure of the Westerwald region yielded different results depending on the statistical approach applied. The Evanno method (STRUCTURE: Bayesian iterative algorithm; Porras-Hurtado et al. 2013) suggests the presence of three population clusters. One cluster included the geographically most distant populations (6 and 9; **Tab. 1**), an assignment exclusively compatible with unauthorized translocation of individuals between the sites. In contrast, the DAPC method (multivariate approach; Jombart et al. 2010) yielded a finer scaled

pattern in which the grouping of populations to clusters was consistent with their spatial arrangement, origin from a common gene pool, and subsequent isolation by distance. As independent evidence for massive translocations among extant populations in the Westerwald is absent, we conclude that the modelled genetic structure by DAPC is probably more reliable than that obtained by the Evanno method.

As the geographical distribution of a species always exceeds an individual´s dispersal capacity, IBD patterns are commonly observed among conspecific populations (Balloux & Lugon-Moulin 2002). The pronounced IBD in the Westerwald populations (steep regression line, **Tab. 6**) emphasizes the interrupted exchange of individuals in this region as predicted for marginal regions. The high frequency of private alleles indicates gene drift effects due to the isolation of populations. Drift effects are probably reinforced by the small population size and the short reproductive lifespan in the investigated populations that decreases an individual´s chance to contribute to the local gene pool (Cayuela et al. 2019a; Hantzschmann & Sinsch 2019).

Amphibians are strongly affected by habitat fragmentation and subsequent isolation of local populations because bottlenecks and high inbreeding levels often reduce fitness (Andersen et al. 2004; Angelone 2010; Apodaca et al. 2012; Ficetola & De Bernardi 2004). Landscape fragmentation in Hesse is considered the main cause of isolation among *B. variegata* populations (Guicking et al. 2017). This is probably true for the Westerwald region as well because during the 19th century the road and rail network expanded parallel to increased clay mining (Schenk 1993). Clay mines provided secondary habitats for yellow-bellied toads, but the expansion of transport network contributed to landscape fragmentation promoting the isolation of these populations. Furthermore, many stepping stone populations between the extant populations have disappeared during the past decades (Veith 1996b). In conclusion, we consider the deep genetic structuring of Westerwald populations as the result of long-term habitat fragmentation and isolation.

Continental scale

The landscape genetics of some European amphibians (e.g., *Hyla arborea*, *Epidalea calamita*; Allentoft et al. 2009; Dufresnes et al. 2013; Rowe et al. 1998; Rowe et al. 2006) are widely compatible with the central-marginal hypothesis, those of others are not (e.g., *Rana latastei*, *Pelobates syriacus*; Garner et al. 2004; Munwes et al. 2010). Putting genetic diversity

estimates (H_e) of marginal *B. variegata* populations into an European amphibian context H_e is similar to that of marginal *H. arborea* populations (Andersen et al. 2004), greater than that of *E. calamita* populations (Allentoft et al. 2009; Rowe et al. 1998), and less than those of *Rana temporaria* (Palo et al. 2003), *Salamandra salamandra* (Najbar et al. 2015), and *Triturus alpestris* populations (Pabijan & Babik 2006). As geographical variables explain only about 40 % of the large-scale variation of AR in *B. variegata* populations, the central-marginal hypothesis does not explain alone the landscape genetics of *B. variegata*. We rather consider demographic processes following the postglacial dispersal to account for a lower genetic diversity at the range margin as also observed in other European amphibians (Beebee & Rowe 2000; Fijarczyk et al. 2011; Garner et al. 2004; Knopp & Merila 2009; Palo et al. 2004; Rowe et al. 2006). Reduced genetic diversity may additionally be a consequence of small effective population size resulting from habitat fragmentation (Arens et al. 2006; Hitchings & Beebee 1997; Marsh et al. 2008; Noël et al. 2006; Reh & Seitz 1990; Tournier 2017; Willi et al. 2006; Zancolli et al. 2014). Consequently, we attribute the marked IBD pattern among populations in the German regions to reduced gene flow caused by landscape fragmentation and demographic processes, whereas the low genetic structuring in Alsace and Trentino suggests a still intact connectivity among populations at the regional scale.

Implications for conservation management

Our study suggests that fragmentation and subsequent isolation of *B. variegata* populations in German regions are the main causes of pronounced genetic structuring and reduced AR. As a reestablishment of connectivity between currently isolated populations over distances larger than 5 km is visionary because of land use and low dispersal capacity of this species, conservation measures should focus on the preservation of remaining populations by improving habitats. (Re)introduction or translocation of specimens, even to populations following a bottleneck, should be considered with caution, as local populations may be adapted to distinct habitat features and pathogens may be spread unintentionally (Orizaola et al. 2010; Taft & Roff 2012; Verhoeven et al. 2010). Improve connectivity between local populations seems to be the crucial factor to mitigate genetic structuring and to increase resilience towards the variation of environmental factors (Frankham et al. 1999; Schön et al. 2011). Small-scale groups of isolated populations (example: the Schmidtenhöhe populations) should be transformed to an interacting meta-population system by stepping stone ponds,

which enable movements of some individuals between neighbouring populations. Thus, informed conservation management of *B. variegata* populations at local scale could consist in the creation of small-scale meta-populations by offering satellite habitats around isolated extant populations, which can be colonized from these nuclei. These meta-population systems would still be isolated from each other, but more resilient to local extinction. On a regional scale, the still existing connectivity among populations such as those in Alsace and Trentino should be conserved to avoid the negative effects of habitat fragmentation currently present in the three German and in the Geneva regions and threatening the persistence of yellow-bellied toads.

General discussion

This study reveals the complex interactions among breeding site choice, larval environment, metamorph size, body condition and locomotor performance in yellow-bellied toads (see Chapter I). As *B. variegata* shows a remarkable philopatry (Barandun & Reyer 1998; Hantzschmann & Sinsch 2019; Kapfberger 1984), the variety of water bodies used for spawning seems to be a by-product of seasonally differing water levels in breeding ponds. This indicates that *B. variegata* uses a temporal rather than a spatial bet-hedging strategy in order to maximise reproductive success in unpredictable habitats (e.g., Barandun & Reyer 1998). Neither aquatic predators, nor competing anuran tadpoles seem to determine breeding site choice, while pond duration is a crucial factor for tadpole survival (Barandun & Reyer 1997a; Hartel et al. 2007). The mesocosm experiments revealed that rather desiccation than predator presence negatively affected larval development and thus are in line with the previous observations. In contrast to predictions of the Wilbur-Collins model and to observations of amphibian species in ephemeral habitats (e.g., Alford & Harris 1988; Denver et al. 1998; Márquez-García et al. 2010; Márquez-García et al. 2009; Merilä et al. 2000; Rudolf & Rödel 2007; Wilbur & Collins 1973), phenotypic plasticity in developmental time and an adaptive trade-off between size at and timing of metamorphosis was absent in *B. variegata*. Thus, the variability in timing of metamorphosis observed in the field is rather a by-product of the temporal bet-hedging strategy than an adaptive timing of metamorphosis in response to pond drying. This is in agreement with previous observations on the effects of water volume, crowding and temperature on the larval developmental rate in *B. variegata* (Böll 2002; Kapfberger 1984).

Carry-over effects of larval environment on life history

The conditions experienced during larval development affected predictably the phenotype of *B. variegata* metamorphs, as size and performance, i.e. the quality of most metamorphs, depended on pond treatment. These carry-over effects from larval to terrestrial stages indicate additional costs for development in ephemeral ponds, potentially increased stress through crowding effects mediated by the corticotropin-releasing hormone (CRH) (Denver 1997; Kapfberger 1984). Similar effects of desiccation on developmental rate and metamorph size of *Epidalea calamita* were ascribed to low genetic variability and counteracting diurnal temperature fluctuations in desiccating ponds (Brady & Griffiths 2000). This study provides

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evidence that in *B. variegata,* locomotor performance is directly related to the conditions experienced during larval development, morphology and behaviour of metamorphs. This is in agreement with observations in other anuran species that suggest a relation between breeding pond conditions, metamorph phenotype and dispersal ability (e.g., Bredeweg et al. 2019; Charbonnier & Vonesh 2015; Goater et al. 1993; John-Alder & Morin 1990; Márquez-García et al. 2009; Tejedo et al. 2000; Yagi & Green 2018). These delayed life-history effects of aquatic stressors thus determine physiological functions and the vulnerability of metamorphs to terrestrial predators and consequently affect survival and fitness in later life stages (e.g., Altwegg & Reyer 2003; Beck & Congdon 2000; Bredeweg et al. 2019; Chelgren et al. 2006; Crespi & Warne 2013; Relyea 2001; Schäfer et al. 2018; Semlitsch et al. 1988; Van Allen et al. 2010). Consequently, conditions experienced during larval development may have strong indirect effects on population dynamics in yellow-bellied toads.

This study presents the first record of carabids preying on *B. variegata* metamorphs (see Chapter II). These observations emphasize the high predation risk posed by invertebrates during the crucial period of life cycle when metamorphs are dispersing from the breeding pond to terrestrial habitats (Toledo 2005). Furthermore, this study provides evidence that timing of and size at metamorphosis determines survival probability of metamorphs, because small individuals were particularly prone to predator attacks by carabids. These observations are in agreement with reports for juvenile anurans and urodeles (e.g., Cabrera-Guzmán et al. 2013; Ovaska & Smith 1988). This role-reversal, i.e. frog and beetle exchange their position as predator and prey, was previously reported for different life stages among carabid and amphibian interactions (e.g., Burbano-Yandi et al. 2018; Carvalho et al. 2012; Elron et al. 2007; Escoriza et al. 2017; Robertson 1989 ; Scholtz & Ralston 2017; Toledo 2005; Wizen & Gasith 2011a; Wizen & Gasith 2011b). In the area studied, carabids large enough to be potential predators were present during the entire reproduction period of *B. variegata* (Taupp et al. 2015). As carabid abundance was also stable during the study, the plasticity of metamorphosis timing seems to be a by-product of the prolonged breeding period rather than an adaptive predator avoidance strategy. In contrast to active hunting reported for some carabid species (e.g., Robertson 1989), predation events during the study were probably the result of random encounters promoted through the experimental design. In the field, however, the risk of *B. variegata* metamorphs to be preyed upon by carabid beetles seems to be rather low. The potential impact of metamorph mortality caused by foraging carabids on local population

dynamics is determined by carry-over effects from larval environment to metamorph phenotype on one side and by predator species composition and abundance in terrestrial habitats on the other side.

The fast-slow continuum of the life-history trait longevity

The range of longevity among *B. variegata* populations differed notably among the local and the continental scale with the variation among cohorts within a population being considerably lower than the entire fast-slow continuum (see chapter III). In contrast to previous assumptions (e.g., Abbühl & Durrer 1998, Di Cerbo et al. 2011, Seidel 1993), extreme longevity seems to be an exception in *B. variegata*. This study provides evidence for the existence of short- and long-lived populations, suggesting that longevity variation in *B. variegata* populations at the continental scale integrates two components. The first component is intrinsic and defines the magnitude of longevity, i.e. a fixed attribute of a population. The second component is extrinsic and determines local variation among cohorts, i.e. environmental stochasticity that affects survival rates. The following discussion will focus on the identification of factors that potentially determine the variability of longevity among yellow-bellied toad populations.

In contrast to current theories of aging, senescence, i.e. age-specific mortality and the timing of its increase (Kirkwood 1977; Kirkwood & Austad 2000; Williams 1957), was absent in *B. variegata* and thus negligible in the variation of local survival rates. In the short-lived *B. variegata* populations, this variation correlated with the among-years variability of local weather, indicating that duration of the activity period and temperature-modulated intensity of activity are crucial factors for survival (e.g., Becker et al. 2018). In contrast, considerably higher survival rates in long-lived *B. variegata* populations were unrelated to local weather variability. A trend to decreasing longevity in the study populations during the past 20 years indicates that climate change may be another source of variation. As the condition index did neither change during the activity period, nor correlate with annual survival rates, either this index may not reflect correctly the nutritional state in *B. variegata*, or the nutritional state of toads is not a major source of mortality and longevity variation. In contrast to predictions of the disposable soma theory of aging and life-history theory (Kirkwood & Austad 2000; Kirkwood & Holliday 1979), growth patterns and age at sexual maturity were stable among short-lived and long-lived populations throughout the range of *B. variegata*. High local pathogen prevalence affects host fitness and survival, truncates age structure in amphibian

populations and may culminate in local extinction events (Adams et al. 2017; Campbell et al. 2018; Finnerty et al. 2018), thus may also determine longevity variation. On a global scale, *Batrachochytrium dendrobatidis (Bd)* and Ranaviruses are among the most detrimental diseases for amphibian populations (e.g., Campbell et al. 2018; Kärvemo et al. 2018; Robinson et al. 2018; Spitzen-van der Sluijs et al. 2017; Spitzen-van der Sluijs et al. 2016; Thorpe et al. 2018; Valenzuela-Sánchez et al. 2018; Watters et al. 2018; Youker-Smith et al. 2018). Though the data obtained in this study is insufficient to exclude substantial disease-driven mortality, pathogens still seem to be a negligible factor affecting longevity, as prevalence of these diseases was low in the investigated short-lived populations. In conclusion, local weather variability was the only extrinsic factor affecting survival and thus longevity in the short-lived populations of *B. variegata*, whereas none of the investigated factors was identified as a source of variation in the long-lived population. In contrast to many amphibian species (Morrison & Hero 2003; Morrison et al. 2004), geographic clines in demographic life-history traits and climate features hence seem to be negligible for the variability of the fast-slow longevity continuum in *B. variegata*.

A new approach to the fast-slow continuum: The palatability hypothesis

Longevity in the field (i.e. 23 years, Hantzschmann et al. 2019) almost reaches the life expectancy of toads in captivity (i.e. 27 years, Mertens 1964; Mertens 1970), indicating that predation is low in long-lived populations. This extraordinary lifespan of more than 20 years in the field is associated with low predation due to camouflage and toxicity (Abbühl & Durrer 1998). In populations exposed to similar predator abundances, improved antipredator protection may reduce extrinsic mortality and thus increase longevity. In many amphibian species, protection is given through chemical defences such as skin toxins that cause unpalatability, decrease predation and increase survival (e.g., Darst et al. 2006; Hettyey et al. 2019; Kowalski et al. 2018). Antipredator protection in *B. variegata* is attributed to bioactive skin secretions that affect the hormonal system, metabolism and the mucosa of predators (Gonzalez et al. 2008; Marenah et al. 2004; Simmaco et al. 2009; Xu & Lai 2015). There is evidence that concentrations of skin toxins can vary considerably among conspecific amphibian populations due to multiple factors such as anthropogenic habitat alteration, geographical clines and nutrition (e.g., Bokony et al. 2019; Coppari et al. 2019; Yotsu-Yamashita et al. 2012; Zhang et al. 2005). So far, variation in skin secretions has not been investigated in *B. variegata*. However, as none of the investigated extrinsic factors seem to

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determine longevity among populations at the continental scale, the position in the fast-slow continuum of longevity may be defined by varying antipredator protection and associated vulnerability to predators. Future research should focus on measurements of skin peptide profiles of *B. variegata* and their biological activity to detect differences among individuals of short-lived and long-lived populations.

Aspects of population structure and dynamics

Hydroregime and precipitation are crucial drivers of reproductive success of amphibians breeding in ephemeral wetlands (Greenberg et al. 2017). At the local scale, reproductive success, recruitment and estimated population size of *B. variegata* were associated with weather variability and varied considerably among years (see chapter IV). This is in agreement with previous studies on *B. variegata* population dynamics (e.g., Marchand 1993, Cayuela et al. 2016b). The study underlines the unpredictable conditions in *B. variegata* habitats, as the characteristics and dynamics of spawning ponds are mainly determined by climatic conditions (Barandun & Reyer 1997a). Drought periods caused high mortality among *B. variegata* larvae in desiccating ponds and negatively affected juvenile recruitment, as previously observed by Barandun & Reyer (1997b). Consequently, population dynamics were affected with a temporal delay. Furthermore, reproduction depended on rainfall and was prolonged after drought periods. This is in accordance with previous observations on the spawning behaviour of *B. variegata* (Barandun & Reyer 1997b). Indeed, timing of precipitation seems to play a major role for the body size of metamorphs about to hibernate and thus may affect survival rates of juveniles. Irrespective of interannual population dynamics, population size remained stable for several years. Hence, populations are likely to persist, as long as adequate reproductive possibilities exist in the spawning habitats (Hartel 2008).

A high site fidelity of individuals was observed in this study. This is in agreement with previous findings on *B. variegata* (Barandun & Reyer 1998; Jacob et al. 2009; Kapfberger 1984; Plytycz & Bigaj 1984). The annual migratory range reported for *B. variegata* individuals is 20 - 732 m (Abbühl & Durrer 1996; Beshkov & Jameson 1980; Hartel 2008; Jacob et al. 2009; Jordan 2012), whereas lifetime dispersal distance covered in long-lived populations is up to 1.2 - 4.5 km (Gollmann & Gollmann 2005; Jehle & Sinsch 2007; Plytycz & Bigaj 1984). In contrast, dispersal distances in the investigated local populations were considerably lower, as distances longer than 500 m seemed impermeable for dispersing toads. Movement capacity in amphibians is associated with density-dependent motivation to disperse, landscape

resistance, the quality of terrestrial habitat, precipitation (moisture) and life expectancy (Abbühl & Durrer 1996; Cayuela et al. 2016c; Gollmann & Gollmann 2005; Hantzschmann et al. 2019; Hartel 2008; Sinsch 2014; Stevens et al. 2006). Differences among these parameters may explain the broad range of reported dispersal distances for *B. variegata* populations. As migration decreases with increasing distance between ponds (Hartel 2008), the isolation observed in the local populations probably occurs due to a lack of stepping stone ponds. In conclusion, population connectivity at the local scale is strongly affected through reduced dispersal ability and results in the isolation of local populations. Dispersal ability may further be limited due to a reduced longevity. As most of the toads reached a maximum longevity of 5 years and maturity in the third year of life, they are likely to reproduce only over a period of 3 years. The reported reproductive strategy of yellow-bellied toads, i.e. long-lived individuals that skip reproduction under harsh conditions (Barandun et al. 1997), hence seems to be absent in the investigated populations. This study suggests that resilience towards changing environments is low in short-lived populations, as multiple unfavourable reproductive periods are not buffered by longevity and may severely affect population persistence.

Conservation genetics

Landscape genetics are applied to assess inter-patch movement and associated connectivity among anuran populations, i.e. gene flow, to unveil genetic structuring of populations in their geographical range and to understand the long-term persistence of species in changing environments (Frei et al. 2016; Waraniak et al. 2019; Zancolli et al. 2014). The investigated landscape genetics of yellow-bellied toads will be discussed in the context of local, regional and continental scale and the predictions on genetic structuring will be evaluated regarding the short-term and long-term isolation of populations (see chapter V). In agreement with a study on populations inhabiting urbanised regions (Tournier 2017), genetic population structure at the local scale mirrored a short-term, i.e. 30 to 40 years, habitat fragmentation, emphasizing the impact of gene drift on small-sized isolated populations. Dry open grasslands without water bodies may present high landscape resistance for yellow-bellied toads at the local scale and thus cause isolation of local populations (Hantzschmann & Sinsch 2019). Similar observations were made for other amphibian species (Jeliazkov et al. 2019). However, distinct loss of alleles due to genetic drift seems to be a long-term process in *B. variegata*, as genetic diversity of local populations was unaffected by the short-term isolation. In conclusion,

random variation of local allele frequencies in small-sized isolated yellow-bellied toad populations leads to genetic structuring within a few decades.

As expected for conspecific populations (Balloux & Lugon-Moulin 2002), an isolation by distance (IBD) pattern was observed at the regional scale. In consistence with the central-marginal hypothesis and as predicted for populations inhabiting marginal regions, the exchange of individuals between populations was limited or interrupted at the regional scale (e.g., Peterman et al. 2013; Gassert et al. 2013). Gene drift effects are probably intensified by the small population size (Cortázar-Chinarro et al. 2017; Luqman et al. 2018). The short reproductive lifespan in the investigated populations may further increase gene drift as the individual´s contribution to the local gene pool decreases (Hantzschmann & Sinsch 2019). Habitat fragmentation and associated isolation of local populations strongly affect amphibians, as reinforced bottlenecks and inbreeding levels may reduce fitness (Andersen et al. 2004; Angelone 2010; Apodaca et al. 2012; Broquet et al. 2010). Besides recent land-use patterns, historic landscape characteristics determine the distribution of amphibian species (Piha et al. 2007a). Therefore, long-term landscape fragmentation is considered as the main cause of the observed isolation and subsequent genetic structuring among *B. variegata* populations at the regional scale. Road networks negatively affect the presence of *B. variegata* populations (Cayuela et al. 2015b). Thus, the expansion of road and rail network in the 19th century in the Westerwald region (Schenk 1993) was probably a major factor contributing to habitat fragmentation and isolation of populations. In addition, many habitats and hence stepping stone populations between the extant populations disappeared during the past decades (Veith 1996a).

The landscape genetics of *B. variegata* at the continental scale are not explained completely by the central-marginal hypothesis, as geographical variables determine only about 40 % of the large-scale variation of allelic richness. In fact, demographic processes following the postglacial expansion seem to account for a lower genetic diversity at the range margin, as observed in other European amphibians (e.g., Beebee & Rowe 2000; Fijarczyk et al. 2011; Garner et al. 2004; Knopp & Merila 2009; Palo et al. 2004; Rowe et al. 2006). In addition, the observed reduced genetic diversity may be a consequence of small effective population size associated with habitat fragmentation (e.g., Arens et al. 2006; Hitchings & Beebee 1997; Marsh et al. 2008; Noël et al. 2006; Reh & Seitz 1990; Tournier 2017; Willi et al. 2006; Zancolli et al. 2014). In conclusion, the isolation by distance pattern among *B. variegata* populations

in Germany (Guicking et al. 2017; Hantzschmann et al. 2020; Weihmann et al. 2019), i.e. at the northern range margin, seems to be a consequence of reduced gene flow due to landscape fragmentation and demographic processes. In contrast, the low genetic structuring in France (Alsace; Vacher & Ursenbacher 2014) and Italy (Trentino; Cornetti 2013) indicates connectivity among populations at the regional scale.

Threats to *Bombina variegata*

This study highlights the ecological consequences of threats such as climate change and habitat fragmentation to which *B. variegata* is exposed. Amphibians may be less capable of adaptation and thus resilient to intense, rapidly emerging threats, especially when combined with environmental changes (Blaustein & Bancroft 2007). Regarding climate change, amphibians are considered to be vulnerable to decreases in water availability associated with global warming (Araujo et al. 2006). Amphibians breeding in ephemeral wetlands are particularly affected, as precipitation is crucial for the onset of breeding and suitable hydroregimes for reproductive success (Greenberg et al. 2017). *Bombina variegata* underwent local extinctions and population declines in western and north-western parts of its range (Kuzmin et al. 2009). Thus, understanding the ecological consequences underlying breeding habitat design is crucial for adequate conservation management (e.g., Cayuela et al. 2011). Population dynamics of *B. variegata* are strongly affected by weather variation which determines survival of metamorphs, breeding probability and dispersal (Cayuela et al. 2016b; Hartel 2008). The life-history strategy of *B. variegata* predicts that long-lived toads compensate loss of spawn within the same season by several spawning occasions and over years through longevity with skipping of reproduction under unfavourable conditions (Abbühl & Durrer 1998; Barandun et al. 1997). Major risks for tadpole survival are high temperatures and desiccation in open habitats (Dittrich et al. 2016). Consequently, climate change and associated prolonged droughts may negatively affect *B. variegata* populations due to reduced reproductive success. In fact, longevity may buffer populations against an increase in climatic variability (Morris et al. 2008). For short-lived *B. variegata* populations, however, compensation of unfavourable breeding conditions through skipping of reproduction seems hardly possible due to reduced reproductive lifespan. Thus, populations with a low longevity may be particularly vulnerable to climate change. In addition, the detrimental effects of habitat fragmentation on *B. variegata* may be reinforced by climate change, as a recolonization after local extinction events is hardly possible in isolated populations.

Implications for conservation management

Conservation measures should thus focus on the preservation of high-quality habitats, e.g. forests, that may mitigate increased temperatures (Dittrich et al. 2016; Scheele et al. 2014). However, as mining areas and quarries are substitute habitats of high conservation value for pioneer species and among the few extant habitats for yellow-bellied toads (Faucher et al. 2017; Flavenot et al. 2015; Veith 1996a), optimising terrestrial and breeding habitats in these areas is crucial for the persistence of *B. variegata* populations. As breeding pond duration is a key factor for recruitment (Barandun & Reyer 1997a; Sinsch et al. 2020), optimisation of breeding habitats should be achieved through the construction of shallow waterbodies with a hydroperiod long enough to enable tadpole development. In short-lived populations, measures should be carried out regularly, i.e. at least on a 3-year-cycle, to reinforce reproductive success. Isolated and genetically less diverse populations, e.g. at the northern range margin, may have a lower evolutionary potential and viability when facing environmental changes (Cortázar-Chinarro et al. 2017; Cushman 2006; Frankham et al. 1999; Zhang et al. 2015). Thus, informed conservation management of *B. variegata* populations at the local scale should improve the connectivity between extant populations to mitigate genetic structuring and to increase resilience towards environmental variation. This may be achieved by offering satellite habitats around isolated extant populations, which can be colonized from these nuclei and may thus establish a small-scale metapopulation system. However, reestablishing the connectivity among isolated *B. variegata* populations at the regional scale is unlikely due to anthropogenic pressure and low dispersal capacity. Conservation measures should instead focus on preserving remaining populations by improving habitats and maintaining still existing connectivity among populations to mitigate the negative effects of habitat fragmentation that threaten the persistence of yellow-bellied toads.

Summary

Amphibian populations are declining worldwide for multiple reasons such as habitat destruction and climate change. An example for an endangered European amphibian is the yellow-bellied toad *Bombina variegata.* Populations have been declining for decades, particularly at the northern and western range margin. One of the extant northern range centres is the Westerwald region in Rhineland-Palatinate, Germany. To implement informed conservation activities on this threatened species, knowledge of its life-history strategy is crucial. This study therefore focused on different developmental stages to test predictions of life-history theory. It addressed (1) developmental, (2) demographic and (3) genetic issues of *Bombina variegata* as a model organism: (1) Carry-over effects from larval environment to terrestrial stages and associated vulnerability to predators were investigated using mesocosm approaches, fitness tests and predation trials. (2) The dynamics and demography of *B. variegata* populations were studied applying a capture-mark-recapture analysis and skeletochronology. The study was complemented through (3) an analysis of genetic diversity and structuring of *B. variegata* populations using 10 microsatellite loci. In order to reveal general patterns and characteristics among *B. variegata* populations, the study focused on three geographical scales: local (i.e. a former military training area), regional (i.e. the Westerwald region) and continental scale (i.e. the geographical range of *B. variegata*). The study revealed carry-over effects of larval environment on metamorph phenotype and behaviour causing variation in fitness in the early terrestrial stage of *B. variegata*. Metamorph size and condition are crucial factors for survival, as small-sized individuals were particularly prone to predator attacks. Yellow-bellied toads show a remarkable fast-slow continuum of the life-history trait longevity. A populations' position within this continuum may be determined by local environmental stochasticity, i.e. an extrinsic source of variation, and the efficiency of chemical antipredator protection, i.e. an intrinsic source of variation. Extreme longevity seems to be an exception in *B. variegata*. Senescence was absent in this study. Weather variability affected reproductive success and thus population dynamics. The dispersal potential was low and short-term fragmentation of populations caused significant genetic differentiation at the local scale. Long-term isolation resulted in increased genetic distance at the regional scale. At the continental scale, populations inhabiting the marginal regions were deeply structured with reduced allelic richness. As consequence of environmental changes, short-lived and isolated *B. variegata* populations at the range margin may face an increased risk of extinction. Conservation measures should thus improve the connectivity among local populations and reinforce annual reproductive success. Further research on the intraspecific variation in *B. variegata* skin toxins is required to reveal potential effects on palatability and thus longevity.

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Zusammenfassung

Weltweit sind die Amphibienbestände rückläufig. Habitatzerstörung und Klimawandel zählen dabei zu den Hauptgefährdungsursachen. Auch die Populationen der europäischen Gelbbauchunke *Bombina variegata* nehmen seit Jahrzenten insbesondere an den westlichen und nördlichen Verbreitungsrändern ab. Ein rezentes nördliches Verbreitungszentrum ist der Westerwald in Rheinland-Pfalz, Deutschland. Zur Optimierung von Schutzmaßnahmen ist die Erforschung der Lebenszyklusstrategie dieser gefährdeten Art essenziell. Diese Studie untersuchte daher die (1) Entwicklung, (2) Demographie und (3) Genetik von *B. variegata* als Modellorganismus: (1) Carry-Over-Effekte der Larvalbedingungen auf spätere Lebensstadien und damit verbundene Anfälligkeit gegenüber Prädatoren wurden mithilfe von Mesokosmen, Fitnesstests und Prädationsversuchen untersucht. (2) Die Dynamik und Altersstruktur von Populationen wurden durch eine Fang-Wiederfang-Analyse und Skelettchronologie ermittelt. Abschließend erfolgte (3) eine Analyse der genetischen Diversität und Populationsstruktur unter Anwendung von 10 Mikrosatelliten-Markern. Diese Studie betrachtete drei geographische Ebenen, um allgemeine Muster und Besonderheiten von Populationen aufzuzeigen: lokale (i.e. ein ehemaliges militärisches Übungsgelände), regionale (i.e. der Westerwald) und kontinentale Ebene (i.e. das Verbreitungsgebiet von *B. variegata*). Die Ergebnisse zeigen einen Effekt des larvalen Umfelds auf Phänotyp sowie Verhalten und folglich auf die Fitness der Metamorphlinge. Dabei sind Größe und Kondition entscheidende Überlebensfaktoren, da kleine Individuen eine vergleichsweise hohe prädationsbedingte Mortalität aufwiesen. Die Lebenserwartung der Gelbbauchunken ist sehr variabel und scheint von extrinsischen Ursachen wie Umweltstochastizität und intrinsischen Faktoren wie der Effektivität des chemischen Prädationsschutzes abhängig zu sein. Extreme Langlebigkeit scheint dabei eine Ausnahme zu sein; Seneszenz wurde nicht nachgewiesen. In den untersuchten Populationen war der Fortpflanzungserfolg und damit auch die Populationsdynamik wetterabhängig und das Ausbreitungspotential gering. Dabei führte kurzfristige Fragmentierung zu signifikanten genetischen Unterschieden zwischen lokalen Populationen und langfristige Isolation zu verstärkter genetischer Distanz auf regionaler Ebene. Im kontinentalen Vergleich waren die Populationen am Rand des Verbreitungsgebietes stark genetisch strukturiert und verarmt. Kurzlebige und isolierte Populationen unterliegen bei veränderten Umweltbedingungen wahrscheinlich einem erhöhten Aussterberisiko. Daher sollten sich Schutzmaßnahmen auf die Verbesserung der Konnektivität lokaler Populationen konzentrieren und einen jährlichen Reproduktionserfolg ermöglichen. Weitere Forschung ist notwendig, um innerartliche Variation der Hautsekrete sowie mögliche Effekte auf Schmackhaftigkeit und damit verbundene Langlebigkeit zu ermitteln.

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