Amphibians in the agricultural landscape

by

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1 Summary

1.1 Summary in German

Im Jahr 2016 wurden in Deutschland 47% der Landesfläche landwirtschaftlich genutzt, womit landwirtschaftliche Flächen das größte Biom in Deutschland darstellen. Etwa 70% dieser landwirtschaftlichen Flächen wurden für den Anbau von Nutzpflanzen verwendet und mit Pestiziden behandelt. Landwirtschaftliche Flächen sind auch ein essentieller Lebensraum für Amphibien. Daher ist eine Exposition der Amphibien gegenüber Agrarchemikalien wie Dünger und Pestiziden wahrscheinlich. Pestizide können für Amphibien hochtoxisch sein und auch bei einem Bruchteil der ursprünglichen Applikationsrate zu hohen Mortalitäten führen.

Um das Pestizidexpositionsrisiko für Amphibien zu evaluieren, wurde das zeitliche Zusammentreffen von Pestizidapplikationen (N = 331) und der Präsenz von Amphibien auf landwirtschaftlichen Flächen für die Rotbauchunke (*Bombina bombina*), den Moorfrosch (*Rana arvalis*), die Knoblauchkröte (*Pelobates fuscus*) und den Kammmolch (*Triturus cristatus*) während der Frühlingswanderung analysiert. In den Jahren 2007 und 2008 waren in dem Untersuchungsgebiet bei Müncheberg (rund 50 km östlich von Berlin) bis zu 80% der wandernden Amphibien auf landwirtschaftlichen Flächen präsent, als Pestizide appliziert wurden. Die Pestizidinterzeption lag für Wintergetreide bei 50-90% und für Winterraps bei 80-90%. Während einer Pestizidapplikation mit 80% Pestizidinterzeption in Winterraps waren 86,6% der reproduzierenden Population der Knoblauchkröte auf landwirtschaftlichen Flächen aktiv. Spät wandernde Arten wie die Rotbauchunke und die Knoblauchkröte waren häufiger während Pestizidapplikationen auf landwirtschaftlichen Flächen aktiv als früh wandernde Arten wie etwa der Moorfrosch. Unter günstigen Bedingungen kam es für früh wandernde Amphibienarten während der Frühlingswanderung zu den Laichgewässern zu keiner Pestizidexposition.

Um den potenziellen Effekt von Pestiziden auf die Population des Grasfrosches (*Rana temporaria*) zu evaluieren, wurde eine Landschaftsgenetikstudie im Weinbaugebiet der Südpfalz durchgeführt. Aufgrund von kleinen Populationen an den Laichgewässern wurden verschiedene Methoden der Beprobung getestet. Weiterhin wurde der neuartige „repeated randomized selection of genotypes“ Ansatz entwickelt, um die genetischen Daten von Geschwistern bei der Ermittlung von

genaueren genetischen Parametern verwenden zu können. Die genetischen Analysen zeigten eine Isolation von drei Laichgesellschaften von der restlichen Metapopulation. Generell wurde eine höhere genetische Differenzierung bei Laichgewässerpaaren im Weinbau (medianer paarweiser F_{ST} = 0,0215 bei 2,34 km und 0,0987 bei 2,39 km Distanz) als bei Laichgewässerpaaren im Pfälzer Wald (medianer paarweiser F_{ST} = 0,0041 bei 5,39 km und 0,0159 bei 9,40 km Distanz) festgestellt.

Die hier präsentierten Studien liefern einen wertvollen Beitrag, um das Expositionsrisiko von Amphibien in ihrer terrestrischen Lebensphase gegenüber Pestiziden sowie mögliche Effekte auf Laichgesellschaften in der Agrarlandschaft besser zu verstehen. Um einheimische Amphibien und ihre (genetische) Diversität langfristig zu erhalten, müssen sowohl das Risikomanagement von Pestiziden als auch die angewendeten landwirtschaftlichen Methoden angepasst werden. Zusätzlich sollte der Naturschutz den Erhalt von bestehenden und die Schaffung von neuen Laichgewässern anstreben um das langfristige Bestehen von Amphibien in der Agrarlandschaft zu ermöglichen.

1.2 Summary in English

With 47% land coverage in 2016, agricultural land was one of the largest terrestrial biomes in Germany. About 70% of the agricultural land was cropped area with associated pesticide applications. Agricultural land also represents an essential habitat for amphibians. Therefore, exposure of amphibians to agrochemicals, such as fertilizers and pesticides, seems likely. Pesticides can be highly toxic for amphibians, even a fraction of the original application rate may result in high amphibian mortality.

To evaluate the potential risk of pesticide exposure for amphibians, the temporal coincidence of amphibian presence on agricultural land and pesticide applications (N = 331) was analyzed for the fire-bellied toad (*Bombina bombina*), moor frog (*Rana arvalis*), spadefoot toad (*Pelobates fuscus*) and crested newt (*Triturus cristatus*) during spring migration. In 2007 and 2008, up to 80% of the migrating amphibians temporally coincided with pesticide applications in the study area of Müncheberg, about 50 km east of Berlin. Pesticide interception by plants ranged between 50 to 90% in winter cereals and 80 to 90% in winter rape. The highest coincidence was observed for the spadefoot toad, where 86.6% of the reproducing population was affected by a single pesticide in winter rape during stem elongation with 80% pesticide interception by plants. Late migrating species, such as the fire-bellied toad and the spadefoot toad, overlapped more with pesticide applications than early migrating species, such as the moor frog, did. Under favorable circumstances, the majority of early migrants may not coincide with the pesticide applications of arable fields during spring migration.

To evaluate the potential effect of pesticide applications on populations of the common frog (*Rana temporaria*), a landscape genetic study was conducted in the vinicultural area of Southern Palatinate. Due to small sample sizes at breeding sites within viniculture, several DNA sampling methods were tested. Furthermore, the novel repeated randomized selection of genotypes approach was developed to utilize genetic data from siblings for more reliable estimates of genetic parameters. Genetic analyses highlighted three of the breeding site populations located in viniculture as isolated from the meta-population. Genetic differentiation among breeding site populations in the viniculture (median pairwise F_{ST}=0.0215 at 2.34 km to 0.0987 at 2.39 km distance) was higher compared to genetic differentiation among breeding site populations in the Palatinate Forest (median pairwise F_{ST}=0.0041 at 5.39 km to 0.0159 at 9.40 km distance).

Summary

The presented studies add valuable information about the risk of pesticide exposure for amphibians in the terrestrial life stage and possible effects of agricultural land on amphibian meta-populations. To conserve endemic amphibian species and their (genetic) diversity in the long run, the risk assessment of pesticides and applied agricultural management measures need to be adjusted to protect amphibians adequately. In addition, other conservation measures such as the creation of new suitable breeding site should be considered to improve connectivity between breeding site populations and ensure the persistence of amphibians in the agricultural land.

2 Introduction

2.1 Background information on amphibians

Amphibians are cold-blooded vertebrates of the orders Gymnophiona (caecilians), Anura (frogs) and Caudata (salamanders). With nearly 7000 living species, Anura is by far the largest order. Caudata only contains about 700 living species, but is more closely related to Anura than to the Gymnophiona. All of the about 207 species of the order Gymnophiona only occur in the tropics of the Americas, Africa and Asia and some tropic islands (Frost, 2017; Stuart et al., 2008).

In Germany, there are 21 endemic amphibian species. Of these, 20 were listed as endangered in at least one federal state of Germany (Kühnel et al., 2009).

Most temperate amphibians live in terrestrial habitats outside the breeding season for hibernation, foraging and growth. These terrestrial habitats can be located hundreds of meters or even kilometers away from breeding ponds (Günther, 1996), dependent on the migration capacity of the amphibian species. Migration of amphibians between land and water is generally connected to breeding activity (Campbell and Reece, 2003). Adults migrate either directly from their hibernation sites or (later in the season) from other terrestrial habitats into suitable water bodies to spawn. The offspring develops from aquatic larvae to air-breathing young amphibians following the process of metamorphosis. After sexual maturity, the young amphibians repeat this life cycle season for season. However, some amphibian species are an exception and may complete their entire live cycles in terrestrial habitats (*Eleutherodactylus coqui*) or are live-bearing (*Eleutherodactylus jasperi*) (Stuart et al., 2008).

Since most amphibians need suitable aquatic and terrestrial habitats at some point in their life cycles, they are considered as an indicator species reflecting the health of both environments (Collins and Storfer, 2003; Conway and Martin, 1993; Hartwell et al., 1998). Also, due to their permeable skin, amphibians are highly sensitive to any changes in their surroundings (e.g. pH) or toxic contaminants (Quaranta et al., 2009). Furthermore, amphibians are an essential member of an ecosystem and it’s food chain, since they serve as predators to invertebrates and are prey for larger organisms (Günther, 1996).

2.2 Global amphibian declines: history and hypotheses

In the early 1970s to 1980s, herpetologists increasingly reported the declines or extinction of amphibian populations in Northern and Central America as well as in Australia (Collins and Storfer, 2003; Stuart et al., 2004). Initially, these reports were received with some skepticism, since amphibian populations often fluctuate widely (Pechmann and Wilbur, 1994), but the population declines were far more widespread and serious than to be expected under normal demographic variation (Pounds et al., 1997). After the First World Congress of Herpetology in 1989 and a U.S. National Research Council Workshop in 1990 (Barinaga, 1990; Wake, 1991), participants consented that an amphibian declines was evidenced, yet no agreement was reached regarding the cause for this declines (Collins and Storfer, 2003). At the Third World Congress of Herpetology in 1997, a call for research focused on the threat of an increased extinction risk of amphibians was verbalized (Storfer, 2000; Wake, 1998), since reports of declining populations persisted.

Knowledge on amphibian declines had greatly improved during the following years, due to new research findings. Based on this new knowledge, three distinctive features were recognized: (1) reports of amphibian population declines and amphibian species' extinctions had increased; (2) the causes occurred over great distances and simultaneously; (3) amphibian declines was also observed in protected areas (Collins and Storfer, 2003; Storfer, 2003; Stuart et al., 2004). The leading hypotheses of the causes for the global amphibian declines were alien species, harvesting of amphibians, climate change (including increased ultraviolet radiation), changes in land use, increased use of agrochemicals and other toxic chemicals as well as emerging infectious diseases (Collins and Storfer, 2003). Alien species may cause declines or even extinctions of native amphibian populations due to introduction of pathogens, competition with or predation on native species (Cruz et al., 2006; Kats and Ferrer, 2003; Knapp et al., 2001). Mass harvesting of amphibians, i.e. for fresh frog legs, was mostly performed during the 20^{Th} century and had a dramatic impact on population sizes. In some regions, estimates suggested that harvesting was solely responsible for at least one third of the observed amphibian declines (Lannoo et al., 1994).

Climate models predicted changes of temperatures and moisture patterns with a speed which was unprecedented (Carey and Alexander, 2003). Amphibians may not be able to adapt to such fast climate changes or migrate to areas with suitable

climates (due to limited dispersal abilities) in time (Collins and Storfer, 2003). In addition to climate change, several studies found damaging effects of UV radiation on amphibians, especially in combination with toxic chemicals (Blaustein et al., 2003; Kiesecker et al., 2001; Vonesh and De la Cruz, 2002), while others did not (Corn and Muths, 2002; Palen et al., 2002).

The impact of changes in (global) land use on amphibian persistence is straightforward, yet complex. The built-up of areas due to the development of traffic infrastructure (causing amphibian road mortality), urbanization, industrialization as well as the expansion and intensification of agriculture caused habitat loss and fragmentation of breeding sites as well as the pollution of amphibian habitats with toxic substances (Gallant et al., 2007; Hartel et al., 2010; Mann et al., 2009; Stuart et al., 2008). Such changes in land use can facilitate the extinction of populations and species on a local or regional scale (Collins and Storfer, 2003) or may hinder amphibians from shifting their distribution to adapt to climate changes (Gascon et al., 2007). The expansion and intensification of agriculture also entails input of a wide variety of agrochemicals, which can be highly toxic to aquatic and terrestrial life stages of amphibians (Brühl et al., 2013; Hooser et al., 2012), into the environment. On the other hand, amphibians can also benefit from land use changes, when suitable artificial breeding sites were created (Mann et al., 2009), for example retention ponds of irrigation and drainage systems (Herzon and Helenius, 2008).

Infectious diseases such as chytridiomycosis (Daszak et al., 2003, 1999) and ranavirus (Gray and Chinchar, 2015) received increased attention since the mid-1990s (chytridiomycosis) and mid-2000s (ranavirus), when researchers made the connection between local amphibian population fluctuations, declines or extinctions and the detection of the diseases. Especially chytridiomycosis, which is caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), is considered as putatively linked to the extirpation of numerous amphibians (Martel et al., 2013; Venesky et al., 2014). Yet, there is no evidence for a causative link between infections with *Bd* and amphibian population declines in Central Europe (Ohst et al., 2011). For *Batrachochytrium salamandrivorans (Bsal),* a fungal pathogen causing chytridiomycosis primarily in salamanders, a causative link between infections and mass die-offs in wild European fire salamanders was established (Martel et al., 2014, 2013). The increasing use of agrochemicals may contribute to the development of multi-resistant fungal pathogens that represent a health threat not only to amphibians

(Fisher et al., 2012). Also, the risk of infection with fungal pathogens increased for amphibians when fungicides were present at ponds (Rohr et al., 2017).

2.3 Current threats for amphibians in Germany

Over the last 30 years, amphibian road mortality, the loss and fragmentation of suitable habitats as well as lethal or sublethal effects of agrochemicals, in particular pesticides, are considered as the major causes for declining amphibian populations in Germany (Bitz and Simon, 1996; Günther, 1996; Kühnel et al., 2009; Petersen et al., 2004; Schütz et al., 2011).

Nature conservation groups such as NABU and BUND are working hard to reduce amphibian road kill, by establishing numerous drift fences alongside roads where amphibians cross (typically to reach their breeding sites). Also, migration of amphibians was considered more and more during road construction in the last decades, by implementing drift fences and tunnels directly into the roads (Haaren and Reich, 2006), which reduced the impact of road mortality from a regional to a global level (Kühnel et al., 2009; Smith and Sutherland, 2014).

2.4 Risk of exposure and toxicity of pesticides

With 47% land coverage in 2016, agricultural land is one of the largest terrestrial biomes in Germany. About 70% of the agricultural land was cropped area with associated pesticide applications. In agricultural landscapes, amphibians have to cross agricultural land during migration from terrestrial to aquatic habitats for reproduction regularly (Berger et al., 2011; Fryday and Thompson, 2012; Günther and Podloucky, 1996) and exposure to pesticides is likely (Becker et al., 2007). Yet, amphibians may not only be exposed to pesticides during their terrestrial life phase, but also during their aquatic life phase, such as adults during reproduction and larvae of most amphibian species until they complete their metamorphosis (Fryday and Thompson, 2012). Therefore, a single individual may be exposed to pesticides during several life stages, in example during larvae development in the aquatic phase and later on, when migrating through terrestrial habitats. Here, the exposure during larvae development may cause impairment for the individual such as deformations or weakened immune system, which might make the individual more susceptible for negative effects of additional exposure events (Salice et al., 2011; Todd et al., 2011).

In the aquatic phase, the toxicity of pesticides for amphibians has been shown by several studies (Ghose et al., 2014; Hooser et al., 2012; McMahon et al., 2011;

Sparling and Fellers, 2009). In the terrestrial phase, pesticides can cause lethal or sub-lethal effects also (Belden et al., 2010; Brühl et al., 2011). The most relevant route of exposure in the terrestrial phase is dermal uptake, since amphibian skin is highly permeable and uptake processes of chemicals are two times faster than in mammals (Quaranta et al., 2009; Van Meter et al., 2014). Uptake via contaminated food is also conceivable, but given the potential for dermal uptake, food intake is less likely to be the major source of pesticide exposure (Smith, 2007).

However, application timing, interception by the crop canopy, and timing of amphibian presence on agricultural land are of great importance for the actual pesticide exposure (Cusaac et al., 2015), yet pesticide management differs between crops and farm, particularly with regard to type, number, amount and date of application of pesticides.

2.5 Amphibians in the European risk assessment of pesticides

The European Directive 91/414/EEC, concerning the admission of plant protection products to the markets, stated that for vertebrate ecotoxicology only fish (for the aquatic environment) and bird as well as mammal (for the terrestrial environment) toxicity data had to be included in the risk assessment. European Directive 91/414/EEC was replaced by Regulation (EC) 1107/2009, introducing an important change: The risk for amphibian and reptile terrestrial stages must be addressed as part of the ecotoxicological risk assessments for plant protection products and for active substances (Regulations (EU) 284/2013 and 283/2013). However to this day, the European Union has not produced any concrete suggestions or guidelines for the risk assessment of plant protection products to amphibians (Aldrich et al., 2016).

A comparison of acute and chronic toxicity data of fish and amphibians showed that amphibians were between 10 to 100 times more sensitive for 4 out of 55 (acute toxicity) and 2 out of 52 (chronic toxicity) chemicals, respectively (Weltje et al., 2013). Since fish were more sensitive for most of the investigated chemicals, Weltje et al. (2013) argued that fish test are appropriate to cover the sensitivity of aquatic vertebrates in current risk assessment procedures and that additional amphibian testing is not necessary. However, Weltje et al. (2013) had to admit that toxic effects of chemicals such as dexamethasone, might not be detected using fish tests. Therefore, any conclusion drawn from the comparison of amphibian and fish sensitivity to chemicals seems daring at the current time, since specific information

on amphibian exposure and variability in sensitivity is not available yet (Aldrich et al., 2016).

Acute oral toxicity of chemicals was compared between amphibians and birds as well as mammals. Due to the limited availability of toxicity data for terrestrial amphibian life stages, only single-dose oral toxicity data for 26 chemicals were compared (Crane et al., 2016). Birds and mammals were more sensitive than amphibians for 25 out of 26 substances, with the exception of dichlorodiphenyltrichloroethane. Therefore Crane et al. (2016) argued that amphibians were protected in current risk assessment procedures for the oral exposure route. Crane et al. (2016) recommend an extended comparison of toxicity data to validate that mammals and birds are adequate toxicity surrogates for amphibians. Yet, other routes of exposure (e.g. dermal uptake) may be even more relevant for amphibians in the terrestrial life stage (Brühl et al., 2011; Fryday and Thompson, 2012).

According to the protection goals of the Regulation (EC) 1107/2009, plant protection products should have no unacceptable effects on the environment and should pose no serious risk to the environment. The detected sensitivity for amphibians to some of the investigated substances raises doubt, if these protection goals are met. Furthermore, the limited information on amphibian sensitivity to plant protection products and relevant exposure routes increase that doubt (Aldrich et al., 2016).

2.6 The development of amphibian landscape genetics

Landscape genetics integrates population genetics, landscape ecology and spatial statistics into one field. The increased availability of spatial data and genetic markers have resulted in great advances in the evaluation of the influence of landscape variables on genetic variation and structure (Storfer et al., 2007). Also, landscape genetic techniques became increasingly accessible due to the rapid adoption of genetic tools (e.g. microsatellite loci) and sophisticated software and techniques (e.g. R, STRUCTURE, GIS) (Richardson et al., 2016).

Although it has been shown that habitat loss and fragmentation as well as changes in land use decrease genetic diversity and increase genetic differentiation among populations (Hitchings and Beebee, 1997; Spear et al., 2005), landscape genetic studies often focused on roads as the driving factor for the observed structuring of populations (Lesbarrères et al., 2006; Safner et al., 2011). Indeed,

roads are strong migration barriers for amphibians (Hels and Buchwald, 2001; Mazerolle et al., 2005). Yet, amphibians do not always have to cross roads with relevant traffic volume when moving among breeding sites. Furthermore, the installation of amphibian tunnels has appeased some of the barrier effects of roads (Glista et al., 2009; Lesbarrères et al., 2004; Woltz et al., 2008).

Agricultural land was primarily seen as a source for breeding site pollution (Marco et al., 1999; Rouse et al., 1999; Taylor et al., 2005). Then, agricultural land was increasingly considered in studies investigating the movement and landscape genetics of amphibians (Arens et al., 2007; Miaud et al., 2000). In general, the number of published landscape genetics studies increased substantially between 2006 to 2010, yet only 11% of the landscape genetic studies included agricultural land as a discrete landscape feature until 2010 (Storfer et al., 2010). In the recent decade, more and more amphibian landscape genetic studies focused on intensive anthropogenic land use and agricultural land in particular (Arens et al., 2007; Frei et al., 2016; Safner et al., 2011).

3 Objectives and thesis layout

The primary objectives addressed in this thesis were to assess the presence of the fire-bellied toad (*Bombina bombina* (Linnaeus, 1761)), moor frog (*Rana arvalis* (Nilsson, 1842)), spadefoot toad (*Pelobates fuscus* (Laurenti, 1768)) and crested newt (*Triturus cristatus* (Laurenti, 1768)) in agricultural land (near Müncheberg (about 50 km east of Berlin), Brandenburg, Germany) during and shortly after pesticide applications as well as to determine the genetic population structure of the common frog (*Rana temporaria)* in a vinicultural area (Southern Palatinate, Rhineland-Palatinate, Germany). These objectives evolved from the need for useful information for the risk assessment on European amphibian species that might be at risk of pesticide exposure (Fryday and Thompson, 2012) and to determine the current status of the common frog (*Rana temporaria)* in intensively managed agricultural land.

In order to accomplish these objectives, four work packages were identified:

- **Amphibian monitoring:** Amphibian activity at breeding sites was monitored during the reproduction period to determine population size and the duration of the reproduction period. Furthermore, period of amphibian spring migration was compared with the timing of pesticide applications to check for temporal coincidence.
    - Brandenburg: Published in Lenhardt et al. (2015)
    - Southern Palatinate: See chapter 4.1.2 and 4.2.2
- **DNA sampling and method establishment:** Several methods for DNA sampling (tissue of eggs and tadpoles as well as buccal, skin and cloaca swabs of adults) were tested to determine the optimal DNA sampling method.
    - Published in Müller et al. (2013) and unpublished data in chapter 4.3
- **Method development for genetic analysis:** Tissue sampling of tadpoles was identified as the optimal DNA sampling method in this study. Due to small population sizes and therefore few clutches per breeding site, we were forced to sample siblings. Sampling siblings can lead to biased results. Therefore, I developed an approach to reduce this bias and to improve the result quality of genetic analyses containing siblings.
    - Published in Lenhardt and Theissinger (2017)

- **Landscape genetics:** In Southern Palatinate, the genetic differentiation of breeding pond populations was analyzed to identify the most relevant landscape elements that explained the observed genetic structure best.
    - Published in Lenhardt et al. (2017)

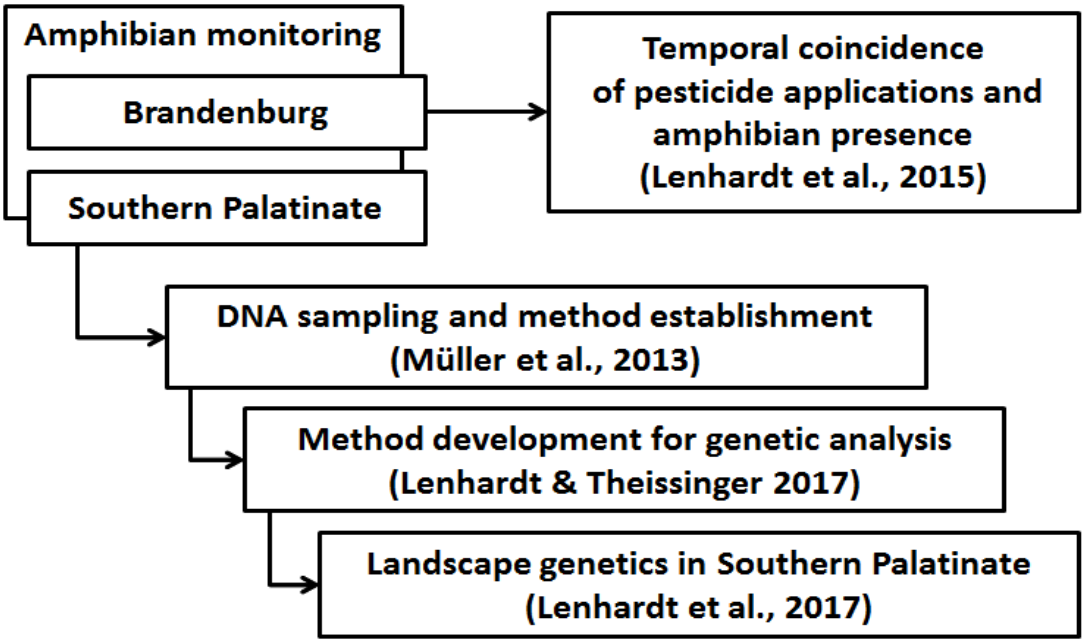

Figure 1 Overview of work packages and associated publications

4 Summarized Material and Methods, summarized Results and Discussion

4.1 Amphibian Monitoring

4.1.1 The fire-bellied toad (*Bombina bombina*), moor frog (*Rana arvalis*), spadefoot toad (*Pelobates fuscus*) and crested newt (Triturus cristatus) in Müncheberg

The amphibian monitoring was conducted by the "Leibniz-Zentrum für Agrarlandschaftsforschung" under the lead of Gert Berger. The fire-bellied toad, moor frog, spadefoot toad and crested newt were monitored from 2007 to 2008 on agricultural land and at breeding sites. In the study area, the total number of trapped migrating adults per migration period ranged from 44 (crested newt) to 1347 (fire-bellied toad). For more detailed information on the exact materials and methods, please see chapter 7.2.3 and (Berger et al., 2011).

4.1.2 The common frog (*Rana temporaria*) in Southern Palatinate

When monitoring amphibians, there are several methods on how to determine the population size of an amphibian species at a breeding site, such as mark and recapture, capture encounter, non-capture encounter, auditory, clutch counting and dip-net fishing (Bower et al., 2014; Efford and Fewster, 2013; Günther, 1996; Karns, 1986; Sutherland et al., 2016). Non-capture encounter and auditory methods were rejected due to inaccuracy.

The common frog is an explosive breeder, which means that the whole breeding pond population breeds within a short time span of just a few days. Also, due to the close spatial proximity of the breeding sites, breeding would start around the same time at all investigated ponds. Given the short breeding period and the limited man power, methods like mark and recapture, dip-net fishing as well as capture encounter were not feasible for this study. Clutch counting was found the most feasible method to determine the population size of the common frog at breeding ponds, since female common frogs lay only one clutch per breading season, typically (Schmeller and Merilä, 2007).

The monitoring of amphibians in Southern Palatinate was focused on water bodies between "Neustadt an der Weinstraße" and "Landau in der Pfalz" as well as west of the motorway A65 (Table 1, Figure 2). In the vinicultural part of the study

area, a total of 23 temporal and permanent waterbodies with amphibian presence was documented (Bischoff, 2008).

Table 1 Overview of monitored amphibian breeding sites with presence of the common frog (*Rana temporaria*) during breeding seasons 2011 to 2014. P1 to P6 were located within viniculture, whereas the remaining ponds were located in the Palatinate Forest. Column Code shows the alternative labeling used in Lenhardt et al. (2013).

Pond/Pop.	Nearest settlement	Code	LAT	LON
P1	Neustadt	H03	49.317766	8.144419
P2	Maikammer	H08	49.307806	8.115214
P3	Edesheim	H18	49.257344	8.106755
P4	Roschbach	H17	49.246470	8.109025
P5	Burrweiler	R13	49.243211	8.083031
P6	Böchingen	H14	49.236416	8.076568
P7	Sankt Martin	-	49.306993	8.063827
P8	Modenbachtal	-	49.271133	8.031846
P9	Eusserthal	-	49.254757	7.962159
P10	Ramstein-Miesenbach	-	49.458626	7.569591

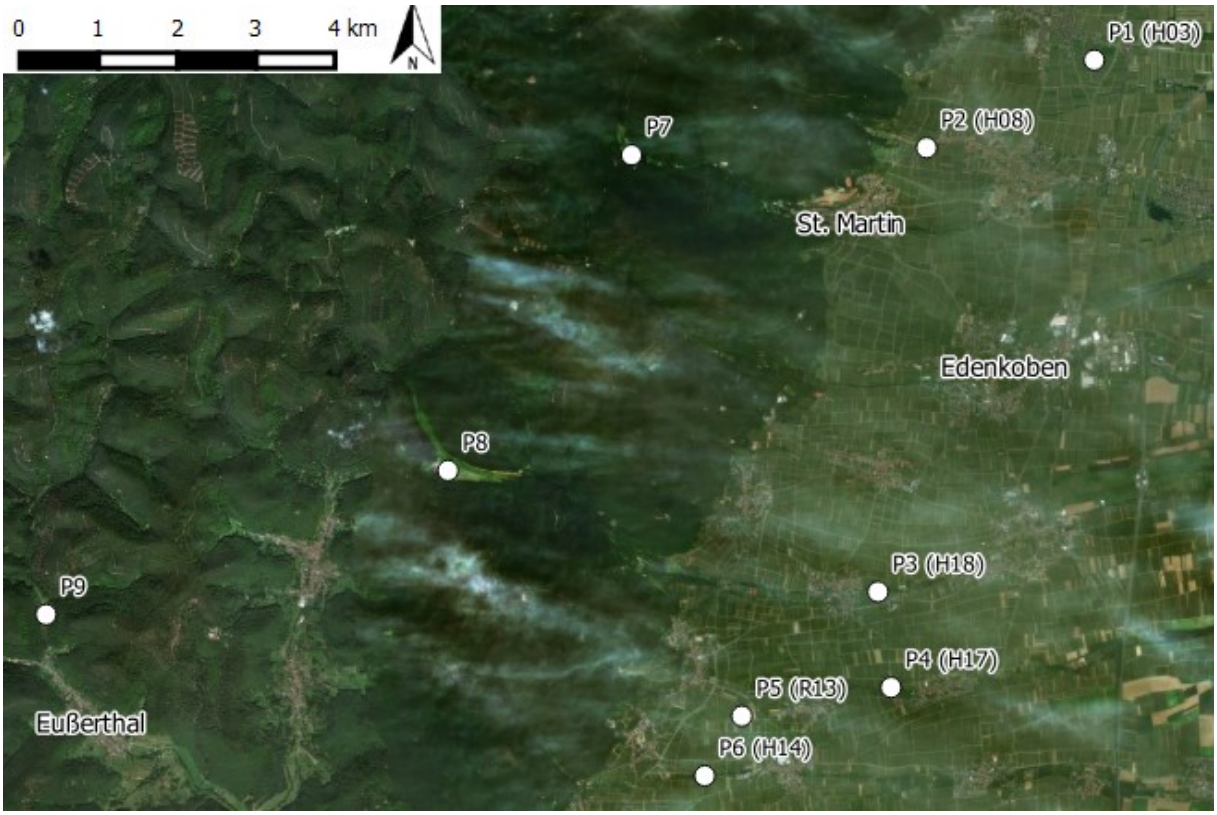

Figure 2 Overview of the study area with breeding sites P1 to P9 between “Neustadt an der Weinstraße” and “Landau in der Pfalz”.

All water bodies were retention ponds and therefore part of the flood prevention and drainage system of the area. At six of these 23 water bodies the common frog was

detected at least once during breeding seasons 2011 to 2014 (P1 to P6). In addition, three breeding ponds (P7 to P9) in the adjacent Palatinate Forest were monitored.

Breeding sites were checked on a daily basis between February 1st and April 30th to determine beginning and end of the breeding season. For 2011 to 2014, the common frog started breeding around mid-February to early March. We observed clutches of the common frog two to seven days earlier in breeding ponds located in the Palatinate Forest than in breeding ponds located in viniculture. Breeding phase was completed in a few days after the first detected clutch. Overall, clutch counts for breeding ponds in viniculture were lower than for breeding ponds in the Palatinate Forest. Also, for all breeding ponds but P2, 2014 was the year with the lowest clutch count (Table 2).

Table 2 Clutch counts at the monitored amphibian breeding sites with presence of the common frog (*Rana temporaria*) during breeding seasons 2011 to 2014 (na = not available).

Pond/Pop.	2011	2012	2013	2014
P1	8	7	7	0
P2	10	12	0	8
P3	9	11	8	0
P4	19	22	8	0
P5	7	8	0	0
P6	8	8	5	0
P7	26	32	27	24
P8	21	25	19	18
P9	> 100	> 100	> 100	> 100
P10	> 100	na	na	na

Although the amphibian monitoring was conducted for only four years and therefore may have a limited explanatory power, the observed clutch count indicates declining population sizes for some of the vinicultural breeding pond populations. When the monitoring window was extended by including data from previous studies during the years 2007 to 2009 (Bischoff, 2008; Bischoff, unpublished data), when about 30 to 60 clutches of the common frog per season were observed at breeding ponds, the general declining trend of the common frog was confirmed in the study area. Also, the observations of declining clutch count falls in line with observations summarized in Schlüpmann et al., (1996), Günther, (1996), Schlüpmann et al., (2004) and Kühnel et al., (2009), where herpetologists found that the number of common frog populations with ten or less clutches increased over time.

At a first glance, the low clutch counts might not sound to alarming, since the common frog is known for small and temporary instable populations and still to persist in the landscape. However, the persistence of small common frog populations can only be possible if several of those small and temporary instable populations can connect to a meta-population on a regular basis (Schlüpmann et al., 1996).

4.2 Temporal coincidence of amphibian presence on agricultural land and pesticide applications

4.2.1 The fire-bellied toad (*Bombina bombina*), moor frog (*Rana arvalis*), spadefoot toad (*Pelobates fuscus*) and crested newt (*Triturus cristatus*) in Müncheberg

Amphibian breeding sites can be located near, next to or even within agricultural land (Berger et al., 2011; Lenhardt et al., 2013). To enter or leave such breeding sites, amphibians often have to cross agricultural land. Therefore, it was not surprising that the presence of amphibians on agricultural land was reported for many species (Fryday and Thompson, 2012). Yet, empirical information on the seasonality use of agricultural land in terms of time spent and distances moved in this habitat were not available.

Partially, the presence of amphibians on agricultural land can be estimated. In spring, amphibians migrate towards the breeding sites for reproduction. The duration of the reproduction phase can be determined by checking amphibian presence at breeding sites. After reproduction, adult amphibians leave the breeding sites and migrate into nearby habitats (Semlitsch, 2008). Therefore, determining the duration of the reproduction phase at the breeding site gives a rough estimate of the potential presence of amphibians on agricultural land prior to and after breeding. Also, after completion of metamorphosis, juveniles leave their breeding sites and move into agricultural land frequently (Berger et al., 2003).

In the study are of Müncheberg, the investigated breeding sites were located inside agricultural land with an average distance of 300 m between breeding site and field edges. So, amphibians definitely had to cross agricultural land to reach the breeding sites. While spring migration towards the breeding sites took place, pesticides were applied on the agricultural land in the study area. Due to the study design with enclosures around the breeding sites, it was possible to obtain a realistic estimate of exposure to pesticides (Lenhardt et al., 2015). Furthermore, the study

design allowed quantification of the exposed individuals and population portion for the investigated amphibian species.

During the analyses of the pesticide use of the monitored farms in the study area, crop-specific pesticide application patterns for winter rape, maize and winter cereals (winter rye, winter barley, winter wheat and triticale) were detected. For winter rape, two delimited application periods for stem elongation and flowering were detected.

In 2007 and 2008, the crested newt, the spadefoot toad and the fire-bellied toad showed temporal coincidence with pesticide applications in winter cereals and winter rape. In 2008, no temporal coincidence for the moor frog was observed, whereas the fire-bellied toad showed temporal coincidence with herbicide application in maize also. Overall, the average population proportion coincident with pesticide applications varied between 0.8 and 74.6%. On average, more than 20% of the captured amphibians coincided with each pesticide application in winter cereals (20.7%) and winter rape (22.8%). Expected crop interception according to FOCUS Working Group (2012) varied between 50 to 90% for winter cereals but remained constant for winter rape (80%). For temporal coincidence of 29% of the total population of the fire-bellied toad with herbicide applications in maize, a crop interception between 0% to 25% can be assumed (FOCUS Working Group, 2012).

In 2007, the highest coincidence with a single herbicide application was detected for the crested newt during stem elongation in winter rape (80% interception) and affected 71.1% of the total population. For insecticides, the highest coincidence with a single application was observed for the fire-bellied toad (39.8% of the total population) during flowering of winter rape (80% interception). In 2008, the highest coincidence with pesticide applications was observed for the spadefoot toad and affected 86.6% (fungicide and insecticide) and 77.9% (herbicides) of the total population (with 50 to 80% interception).

Early breeders like the moor frog (*Rana arvalis*) and the crested newt may not coincide with the pesticide treatments of agricultural land during spring migration under favorable circumstances. Late migration species like the fire-bellied toad and the spadefoot toad are more likely to be exposed to pesticides during spring migration. Over all species, up to 80% of the migrating amphibians temporally coincided with pesticide applications on agricultural land during spring migration. 86 % of the population of the spadefoot toad was exposed to a pesticide during a

single application (Lenhardt et al., 2015). Yet, with exception of an atypical herbicide application in maize, all pesticide applications were conducted during crop growth stages stem elongation and flowering (BBCH Working Group, 2001; Lenhardt et al., 2015), resulting in a pesticide interception by plants of 50 to 90% (FOCUS Working Group, 2012).

The study provides the assessment of potential temporal coincidences of adult amphibians, migrating from hibernation sites into breeding ponds, and pesticide applications on agricultural land during spring migration. Furthermore, it was shown that for most of the coincident pesticide applications, plant interception in winter cereals and winter rape is high (50 to 90%) during spring migration (Lenhardt et al., 2015). Despite this high interception during migration, amphibians can be harmed by pesticides because 10% of the typical application rate of some pesticides can cause lethal effects (Belden et al., 2010; Brühl et al., 2013). Also, amphibians may encounter pesticide applications in combination with other stressors such as predators, competition, pathogens and climate change (Blaustein et al., 2003; Hof et al., 2011). Sublethal effects of pesticides may make amphibians more susceptible to such stressors and therefore reduce the survivability or reproduction capabilities of exposed amphibian individuals (Rohr et al., 2017; Woodley et al., 2015).

Amphibians living in spatial proximity to agriculture may develop tolerances to some of the applied pesticides, even though circumstances and limitations of such tolerances remain vague (Cothran et al., 2013; Hua et al., 2013). It has been shown that aquatic invertebrates, that adapted to pesticide contamination, have a reduced genetic diversity (Coors et al., 2009). A reduced genetic diversity could make amphibians even more vulnerable to other or changing stressors (Lesbarrères et al., 2005).

Since the study was limited to spring migration, the temporal coincidence of amphibian presence on agricultural land and pesticide applications after breeding and during summer remains unclear. However, additional pesticide exposure on agricultural land cannot be excluded for adult amphibians after breeding. Adults and juveniles emigrating from the investigated breeding sites in Müncheberg would definitely have to move over agricultural land for about 300 m. With an assumed daily migration range of 100 m (Berger et al., 2012), juveniles would be at risk to encounter a pesticide application for at least three days. When considering that juveniles may have a smaller daily migration range than adults (see 4.2.3), the timespan for

potential pesticide exposure increases. If juveniles stay in the agricultural land surrounding their natal ponds due to foraging or other activities, the risk to encounter a pesticide application increases further.

4.2.2 The common frog (*Rana temporaria*) in Southern Palatinate

As an early breeder, the common frog completes his reproduction phase before pesticide applications were performed in the vinicultures of Southern Palatinate (Lenhardt et al., 2013). Therefore, pesticide exposure was unlikely for adult common frogs during spring migration, but may be possible during summer, if viniculture was used as a summer habitat by adults. Juveniles approximately emigrated from breeding sites during June and August, based on the observed reproduction period and estimated tadpole development. During July and August, multiple fungicide applications were conducted in the viniculture of Southern Palatinate (Lenhardt et al., 2013) Therefore, the pesticide exposure of juvenile common frogs was likely. Since detailed information on movement patterns and habitat use of emigrating juveniles were not available, an accurate quantification pesticide exposure was not possible.

4.2.3 General discussion of amphibian movement, migration and connectivity

Since most endemic amphibians have a biphasic life cycle with an aquatic (reproduction and larvae phase) and terrestrial (juveniles and adults) stage, movements to and from breeding sites are crucial for the survival of breeding site populations. Movements among breeding site populations at the landscape or regional scale are crucial for recolonization after local extinction and maintenance of meta-populations (Marsh and Trenham, 2001). Therefore, understanding and distinguishing amphibian movement at breeding site population and meta-population level is essential for effective management and conservation of amphibians.

Amphibian movements can be differentiated in migration and dispersal (Semlitsch, 2008). Amphibian migration includes the movement of adults toward and away from aquatic breeding sites as well as movements between foraging habitat, summer refugia as well as hibernation sites and is annually repeated (Günther, 1996; Lamoureux et al., 2002; Sinsch, 1988). The daily migration distance of adult amphibians varies between few to several hundred meters (Jehle and Arntzen, 2000; Miaud et al., 2000). Investigations on adult crested newts and fire-bellied toads showed average daily migration distances between 93 m to 149 m in the study area of Müncheberg, Germany, about 50 km east of Berlin (Berger et al., 2012).

Therefore, an average daily migration distance of 100 m was defined while investigating the temporal coincidence amphibian migration and pesticide applications in the study area. Combined with an average distance of 300 m from field edges to breeding ponds (Berger et al., 2012), a time span of three days from entering the field to reaching the breeding pond was assumed.

Considering only the migration of the field edge to breeding pond was an optimistic approach, assuming that adult amphibians would not have to cross any additional fields on their way from the hibernation site to the breeding ponds. If amphibians would have to cross any additional fields, the potential for temporal coincidence of amphibian presence on agricultural land and pesticide applications would increase. Yet, the exact migration route from hibernation site to field edge was unknown. Also, literature on amphibian preferences for migration routes was inconclusive, since some authors suggest amphibian individuals move in approximately straight lines to target sites (Bulger et al., 2003; Semlitsch, 2008), whereas other authors suggest avoidance or preference for particular landscape feature or vegetation type (Birchfield and Deters, 2005; Müllner, 2001; Searcy et al., 2013; Semlitsch, 2008; Todd et al., 2009). Therefore the amphibian migration before reaching the field edge was legitimately excluded from the study.

During post-breeding migration, adult amphibians typically make several consecutive terrestrial movements away from the breeding site. Amphibians often stay about 300 m next to the breeding site (Semlitsch and Bodie, 2003), while using both agricultural and non-agricultural land for foraging and as hideouts (Becker et al., 2007; Crawford and Semlitsch, 2007). After reproduction, adult amphibians were also detected on agricultural land in the study area of Müncheberg. Yet, the study design with fences, traps and enclosures was not suitable to quantify population shares of amphibians exposed to pesticide applications during post-breeding migration. Therefore the study Lenhardt et al. (2015) was limited to the temporal coincidence of amphibian presence on agricultural land and pesticide applications during spring migration directed towards the breeding sites.

In contrast to the annually repeated amphibian migration between hibernation site, breeding site and summer habitat, amphibian dispersal is characterized as a unidirectional movement away from the pond of birth and local breeding site population towards other breeding site populations or not yet colonized breeding sites (Semlitsch, 2008). Amphibians can disperse as juveniles after emigration from

the natal pond and as adults after breeding, typically. Adult amphibian dispersal is limited to a small portion of the local breeding site population (Sinsch, 2014), usually less than 10% (Gamble et al., 2007; Reading et al., 1991). Therefore, connectivity between local breeding site populations is predominantly effected through juvenile dispersal (Petranka et al., 2004; Preisser et al., 2000). Also, juvenile amphibians were reported to move greater distances than adults during dispersal (Calhoun et al., 2005; Müllner, 2001; Preisser et al., 2000).

Yet, it is argued that juvenile dispersal takes places over multiple phases and seasons (Pittman et al., 2014; Semlitsch, 2008), since juvenile amphibians should have less locomotor capacity than adults due to smaller body mass and size (Beck and Congdon, 2000). Also, habitat loss and fragmentation reduces survival as well as the dispersal abilities of amphibians (Cushman, 2006; Janin et al., 2012; Rothermel, 2004; Stevens et al., 2006a, 2004). Especially farmed landscapes may form a larger barrier for juveniles than for adults (Vos et al., 2007). Nonetheless, juveniles play a key role in the connectivity (genetic exchange), persistence and development of breeding site populations (Cushman, 2006; Schmidt, 2011).

Sinsch (2014) suggests that the currently established migration and dispersal distances may lead to an underestimation of the actual movement capacities of amphibian species. In his opinion, many amphibian movements remain unnoticed when the position of an amphibian individual is only checked once per day. Also, long-distance displacement by the current of streams may be more frequent than previously thought during breeding migrations (Sinsch, 2014). Underestimated movement capacities of amphibians could explain discrepancies between assumed amphibian dispersal distances and estimated gene flow. Since long-distance displacement between all breeding sites (with exception of P10) investigated in Lenhardt et al. (2017) would be possible, all population pairs were included in the analysis of genetic differentiation.

Migration and dispersal of juvenile as well as adult amphibians are often triggered by rain events and happen almost exclusively at night (Hels and Buchwald, 2001; Todd and Winne, 2006). Once movement has begun, amphibians may continue migration and dispersal during daylight hours (Wells, 2010). In case of an extend time period with unfavorable conditions for movement (e.g. low soil humidity, dry climate), amphibians may shifted to movement during daylight generally (Sinsch,

1988). Compared to overnight, the risk of road mortality and pesticide exposure is increased during daylight, due to higher human activity.

4.3 DNA sampling and method establishment

A well-designed sampling scheme is critical for obtaining accurate results from population genetic studies and for drawing appropriate conclusions about the genetic structure of the investigated populations (Goldberg and Waits, 2010; Storfer et al., 2007). For genetic studies with amphibians, the first sampling design decision has to be the choice between the sampling of larvae and adult individuals. Both approaches have advantages and drawbacks, which need to be weighed against each other, to select the best sampling method for the designated study area and species.

With swabbing, a non-invasive method for DNA sampling was investigated with adult amphibians in a pilot study (Müller et al., 2013). The high salt extraction method (Aljanabi and Martinez, 1997) was found to be a reliable and cheap alternative to commercial kits. A genotyping success of 71.8% for buccal swabs, 41.4% for skin swabs and 51.9 % for cloaca swabs over all loci was achieved. A genotyping error rate of only 0.4% for buccal swabs was detected, whereas skin and cloaca swabs exhibited genotyping errors of 12.3 and 14.1%, respectively. The genotyping errors in skin and cloaca swabs resulted from contaminations with DNA of foreign individuals. Such contaminations are likely, when males are grasping the females during mating season.

In general, adult sampling should be preferred, since the amphibian species of the sampled individuals can be determined more reliable than larvae. Also, sampling adult amphibians is typically less biased than larvae, since larvae represent only a small proportion of the total effective population since not every adult may breed every season (Niemelä et al., 2006). The DNA yield and quality achieved with buccal swabs was good. Yet, the sampling of buccal swabs requires an intensive handling of the adult amphibian individuals such as separating paired individuals and opening the amphibian mouth with force, which can easily result in inquiries. The risks of injuries of buccal swabs as well as the inadequate genotyping success and genotyping error rate of skin and cloaca swabs were strong arguments against adult sampling. Furthermore, for most of the breeding ponds in the viniculture, all adult individuals at the breeding site would have to be caught to reach a reliable sample size of at least 14 individuals. Therefore, sampling adults for DNA would mean to disturb all reproducing individuals.

In population genetic studies on amphibians (e.g., Ficetola et al., 2008; Morgan et al., 2008), fishes (e.g., Hansen et al. 1997) or insects (e.g., Taubmann et al. 2011) eggs or larvae are often sampled instead of adults due to their higher accessibility and abundance. Since clutch counting was already the method of choice for the amphibian monitoring, eggs from clutches were collected and hatched to an early tadpole development stage (Gosner stages 20–25). Then, the DNA was extracted from the tissue of the hatched tadpoles. For tadpole samples, a genotyping success of 92.9% over all loci was achieved with the high salt extraction method.

4.4 Method development for genetic analysis: Repeated randomized selection of genotypes

Due to the easy accessibility, sampling eggs and hatching them for DNA extraction was a practical approach in the field. Yet, sampling eggs in combination with few clutches (less than ten) per breeding site in the viniculture introduced problems for the genetic data analysis, when more than one individual per clutch was considered for analysis. Siblings in the data set may introduce bias and the removal (or at least the reduction) of full-siblings was the recommended ‘best practice’ to resolve such bias (Goldberg and Waits, 2010; Olafsson et al., 2014; Peterman et al., 2016; Whiteley et al., 2012). The removal of full-siblings in the data set was accomplished by selecting only one individual per clutch (Whiteley et al., 2014). The removal would result in a small sample size of seven to ten individuals per breeding sites for the investigated ponds in the viniculture.

In theory, resampling the data to form multiple random subsets would be unnecessary, since full-siblings are highly genetically similar and resampled subsets should produce similar results (Whiteley et al., 2014). Yet, a test with 100,000 subsets for two breeding pond populations showed, that the results for the pairwise F_{ST} value varied with a range of 0.1 between the lowest (0.0041) and highest (0.1041) estimated value (median pairwise F_{ST} value 0.0559; between P1 and P3). The test showed that the selection process of the individuals from the clutch is a critical step, which affects the estimate of genetic parameters when one individual is selected over another, especially in small populations. The variation of the estimated genetic parameter in the subsets may be the result of half-siblings in the clutch due to post-mating clutch piracy in amphibians (Vieites et al., 2004). Therefore, the aggressive purging of siblings from the data set may resulted in a loss of the genetic

data of the half siblings. A result from a single subset may be an outlier and does not reflect the real underlying genetic signal.

A solution to this problem would be to only remove full-siblings from the data set, but keep half-siblings. Yet, methods for sibling inference are not infallible, particularly for identification of half-siblings (Waples and Anderson, 2017). Also, some genetic analysis (e.g. STRUCTURE) would still be affected by the presence of family structure in the data set (Anderson and Dunham, 2008; Rodriguez-Ramilo and Wang, 2012). The repeated randomized selection of genotypes approach meets the requirement that no full-siblings should be contained in a sub set, yet includes all the available individuals in the overall results. Therefore, the results generated with the repeated randomized selection of genotypes approach reflect the real underlying genetic signal in the best possible way. Each subset of the randomized selection of genotypes approach could be analyzed for half-siblings. With the removal of these half-siblings from the subsets, all family structure could be purged from the subset, which could improve the quality of the estimated genetic parameters further. Due to already small population size, the removal of half-siblings from the subsets was not an option in this study and therefore not investigated.

With the repeated randomized selection of genotypes approach, it was demonstrated that siblings can be utilized to produce reliable estimates for H_E, H_O, F_{IS}, R_{IS} as well as pairwise F_{ST} and R_{ST} for sample sizes smaller than ten genotypes per subpopulation. For all of the investigated genetic estimates, a wider range between minimum and maximum values was observed when sample size was reduced, whereas median values remained stable. 100,000 repetitions were found to be robust to cover the whole possible range of values for the investigated genetic estimates. Also, it was demonstrated that selecting only one sibling per sibling group for genetic analysis, which is the currently advised practice, may produce unreliable results, especially when sample size is reduced.

Overall, the handling of siblings in population genetic data sets is a complex problem with no simple solution. Also, a too aggressive removal of siblings from data sets has the potential to produce biased results for genetic estimators (Waples and Anderson, 2017). By variating the number of selected individuals per clutch, or generally speaking family group, the repeated randomized selection of genotypes approach can be modified to meet upcoming requirements for the inclusion of more siblings in the subsets.

4.5 Landscape genetics

4.5.1 The common frog (*Rana temporaria*) in Southern Palatinate

Agricultural land moved into the focus of landscape genetics studies on amphibians (Arens et al., 2007; Frei et al., 2016; Safner et al., 2011). In Arens et al. (2007), Safner et al. (2011) and Frei et al. (2016), all agricultural land was seen as one habitat type, independent of the actual crops and applied management measures. Seeing all agricultural land as one habitat type was a strong simplification of the landscape, since type, timing and number of agricultural management measures such as tillage operations and pesticide as well as fertilizer applications vary with crop (Berger et al., 2012; Lenhardt et al., 2017, 2015). For agricultural areas with monocultures, like the vinicultures in Southern Palatine, it can be assumed that agricultural management measures are more homogenous due to uniform plant growth and widely homogenous weather conditions (Lenhardt, 2011).

The analysis of the genetic data showed structuring within the investigated breeding pond populations of the common frog. Overall, the number of sampled alleles for breeding site populations located in the Palatinate Forest (N = 172) was slightly higher than for breeding site populations located in viniculture (N = 162). STRUCTURE found dominant clusters in the breeding pond populations P1, P2 and P4 (all three located in viniculture), and one in the remaining populations (see Figure 10 and Figure 11). With exception of P1 (H_E = 0.852), all pond populations located in viniculture showed lower levels of heterozygosity over all loci (H_E = 0.663 to 0.776) than populations located in the Palatinate Forest (P7 to P10; H_E = 0.788 to 0.840). We detected no isolation by distance based on linear geographic distance, roads and associated traffic intensity. Habitat types such as viniculture and forest showed significant isolation by distance, indicating that they are the most relevant habitat types to explain the structuring of breeding pond populations in the study area.

Generally, genetic differentiation among breeding pond populations in viniculture was higher compared to breeding pond population pairs in the Palatinate Forest (Table 12), despite close proximity of the breeding ponds. The highest genetic differentiation was observed between breeding pond populations P1 and P2 with a linear geographic distance of less than 2.5 km (MPF = 0.0987 and MPR = 0.1137). Overall, viniculture was identified as the most dominant agricultural land use (Lenhardt, 2011) as well as on of the most relevant landscape features to explain the genetic differentiation among the investigate breeding site populations (Lenhardt et

al., 2017). Furthermore, populations at breeding ponds in the viniculture with a direct connection to the Palatinate Forest by permanent or seasonal streams exhibited lower genetic differentiation to forest pond populations (P3, P5 and P6) as compared to vinicultural pond populations not connected to the forest (P1, P2 and P4). The lower genetic differentiation at breeding sites in the viniculture with a connection to the Palatinate Forest by permanent or seasonal streams indicates the importance of streams, including the adjacent riparian vegetation, for the genetic connectivity in amphibian breeding pond populations (see 7.4). In the study area of Lenhardt et al. (2017), streams could be used for passive displacement (Sinsch, 2014), allowing one-directional genetic exchange over long distances which would otherwise exceed the dispersal capacity of the common frog. Passive displacement of amphibians by stream may also be the result of anthropogenic interaction with amphibian clutches, as observed in 2012 (Lenhardt et al., 2017).

In line with the findings of Lenhardt et al. (2017), several other studies showed negative effects of intensive agriculture on the occurrence, abundance and genetic diversity of amphibians on a regional and national scale (Johansson et al., 2005; Trochet et al., 2016; Youngquist et al., 2016). The detected genetic differentiation for the common frog in the viniculture of Southern Palatinate was similar to the detected genetic differentiation for the moor frog (F_{ST} = 0.06; Noord-Brabant, Netherlands) and the common frog (F_{ST} = 0.024 to 0.193; Chambery, France) in areas with intensive agriculture (Arens et al., 2007; Safner et al., 2011).

Typically, up to 12 fungicide applications per year are applied in vineyards of Southern Palatinate during early May and mid-August (Lenhardt et al., 2013; Roßberg, 2009). Fungicide applications are often applied few days before or after rain events of more than 3 mm precipitation (Lenhardt et al., 2013). Such rain events can also trigger amphibian migration or general amphibian activity (Baldwin et al., 2006; Rothermel, 2004). During June and mid-August, juvenile common frogs migrate away from the spawning waters and adult common frogs are in their terrestrial life stage. Therefore, the spatial and temporal overlap of amphibians and applied fungicides is very likely.

Pesticide, and especially fungicide applications are the most frequent management measure in viniculture (Lenhardt et al., 2013). Pesticides can cause high mortalities in amphibians (Belden et al., 2010; Brühl et al., 2013; Wagner et al.,

2017). Therefore, pesticides may have a major impact on amphibian dispersal and on genetic exchange between breeding sites.

In a study area where agricultural land was less than 12% of the area, no impact of agricultural land use on the genetic differentiation of amphibians was observed (Stevens et al., 2006b). In areas, where agriculture is not a dominant landscape feature, negative effects on genetic diversity and differentiation of amphibians may be mitigated when amphibians can avoid agricultural land. However, in areas where intensive agriculture is dominant, avoidance of agricultural land is not practicable for amphibians, since natural habitats are fragmented and breeding sites are completely surrounded by agriculture (Berger et al., 2011; Lenhardt et al., 2013).

In the study area of Lenhardt et al. (2017), breeding sites were between several hundred meters to few kilometers apart from neighboring breeding sites. In addition, the study area contained numerous permanent as well as temporal standing and flowing waters (Lenhardt, 2011). In Frei et al. (2016), breeding sites were located in agricultural land and distances between neighboring breeding sites were also several hundred meters to few kilometers. Yet, Frei et al. (2016) did not detect genetically isolated populations, although a trend for genetic differentiation was observed.

4.5.2 Amphibian persistence in agricultural land and the role of pesticides

Although the number and size of amphibian breeding site populations is declining globally (Stuart et al., 2008), many species have been able to persist in agricultural landscapes so far. It is argued that in course of agricultural intensification, new breeding sites and wetland habitats would be provided due to the establishment of flood and rain retention areas, drainage systems, irrigation channels and dams (Mann et al., 2009). Amphibian species that can colonize and utilize such new breeding sites and habitats are able to persist as meta-populations (Herzon and Helenius, 2008; Knutson et al., 2004). Also, while amphibians migrate and disperse through the agricultural landscape (Fryday and Thompson, 2012), they can use agricultural land for foraging (Miaud and Sanuy, 2005; Oldham and Swan, 1992).

Negative effects of habitat loss and fragmentation on the persistence of amphibian populations may be mitigated or compensated. Yet, agricultural land introduces additional stressors for amphibians such as tillage operations and pesticide as well as fertilizer applications (Berger et al., 2012; Dürr et al., 1999; Lenhardt et al., 2015). Especially pesticide applications are of great importance, due to their high toxicity for amphibians and the frequent application (Brühl et al., 2013;

Lenhardt et al., 2013). Currently and recently used pesticides can be detected in the soil of agricultural land up to several months after their last application (Hvězdová et al., 2018). Therefore, amphibians moving over or foraging in agricultural land may be permanently exposed to low concentrations (< 0.2 mg/kg) of pesticides.

The evident pesticide exposure of amphibians on agricultural land endorses the relevance of pesticides in the examination of amphibian persistence (Lenhardt et al., 2015). To date, there are no adequate techniques available to directly investigate the effect of pesticides on amphibian meta-populations or amphibian persistence in agriculture. Current landscape genetic studies can include the effect of pesticides only by considering agricultural land as a surrogate for all applied agricultural management measures.

Studies on amphibian landscape genetics and movement behavior have shown the positive effect of a dense breeding site and wetland network for amphibian persistence (Fortuna et al., 2006; Frei et al., 2016; Gómez-Rodríguez et al., 2009; Knutson et al., 2004). So, it remains unclear how dense such a network has to be to allow amphibian persistence in the agricultural land. Also, most studies on amphibian landscape genetics and movement behavior in agricultural land remain vague, when it comes to the cultivated crops and applied agricultural management measures in the investigated study areas. Therefore, the comparison of such studies is difficult, since agricultural management measures vary with crop and region. Including detailed information about crops and type as well as timing of agricultural management measures would allow a more differentiated interpretation of study results in the future.

Despite all efforts in amphibian conservation, the persistence of amphibians in agricultural land seems in limbo, since meta-populations lose local amphibian populations at an average rate of 3.79% per year (Grant et al., 2016).

5 Conclusion

The exposure of amphibians to pesticides is dependent on pesticide applications, the presence of amphibians on the agricultural land during or after the pesticide application as well as the pesticide interception by the crop. In this thesis, the temporal coincidence of amphibian presence on agricultural land and pesticide applications has been proven for four amphibian species. It has been shown that up to 80% of all migrating amphibians encountered at least one pesticide application during spring migration. The average population proportion coincident with pesticide applications varied between 0.8 and 74.6% over all investigated amphibian species and pesticide types. On average, more than 20% of the captured amphibians coincided with each pesticide application. Furthermore, it has been shown that a single application can affected up to 86.6% of the reproducing population of a single amphibian species. Although, a pesticide interception by crops can be up to 90%, even the remaining 10% of the initial application rate can cause high amphibian mortality. The evident amphibian presence on agricultural land during pesticide applications highlights the importance of dermal uptake as an exposure route that is not covered adequately in the current risk assessment of pesticides. Until amphibians are covered adequately, avoiding pesticide applications during major amphibian migrations may be advisable.

To be able to investigate the genetic structure of the common frog, a novel repeated randomized selection of genotypes approach was developed to obtain reliable estimates of genetic parameters for populations with small sizes by utilizing data of siblings. The approach can be directly applied to any other species when full-siblingship only exists within the sampled unit (e.g. eggs of clutch). Otherwise the approach has to be extended with a test for full-siblingship, to avoid bias due to full-siblings in the subsets. It has been shown that the repeated randomized selection of genotypes approach produces more reliable estimates of genetic parameters than a single random selection approach, since the single random selection approach leaves genetic information of unselected genotypes unconsidered.

The structuring of breeding site populations as well as a trend for declining population sizes at individual breeding sites has been shown for the meta-population of the common frog in the viniculture of Southern Palatinate. The effect of pesticides on the meta-population of the common frog was only investigated indirectly by

assessing the effect of viniculture as a surrogate for all associated effects. Yet, due to timing, frequency and toxicity of the applied substances on amphibians, pesticides have to be considered as the main driving factor for the observed structuring and declining population sizes in the vinicultural area of Southern Palatinate.

Type, timing and number of pesticide applications (and other agricultural management measures such as tillage operations and fertilizer applications) varies with crop and region. Also, timing of amphibian migration and dispersal varies with species and region. Therefore, amphibian conservation strategies cannot be generalized but must be developed under the consideration of regional circumstances and amphibian species. Based on the available ecotoxicological data, the effect of pesticides on amphibian individuals and populations cannot be assessed adequately. Therefore, it may be advisable to reduce or avoid pesticide applications during amphibian migration phases and to mitigate pesticide contamination of amphibian breeding ponds. I recommend further research on the impact of pesticides on amphibian individuals and populations in agricultural landscapes, to expand the ecotoxicological data on amphibians and advance the amphibian risk assessment for plant protection products.

6 Outlook: The future of amphibians

Around three decades ago, the decline of amphibians was proposed as a global phenomenon. Although researchers have identified multiple drivers of the amphibian declines, there is little progress in halting or reversing the trend. Recent research suggests that the effects of the identified drivers of amphibian declines are variable at regional scales and therefore conservation strategies cannot be generalized (Grant et al., 2016).

Although drivers of amphibian declines are variable, the destruction and contamination of breeding sites is highly critical (Arntzen et al., 2017; Ulrich et al., 2015). Latest predictions suggest that the number of occupied sites for amphibian species will decrease about 50% within the next two decades (Grant et al., 2016), while breeding ponds get lost at a rate of 3.5% per year (Arntzen et al., 2017; Curado et al., 2011). Indeed, the conservation of existing and the creation of new breeding sites, to providing a dense network of breeding sites and other waterbodies, has priority in amphibian conservation (Frei et al., 2016). However, empirical data on the required density of breeding site networks to support the persistence of amphibian species is not available to date.

A dense network of breeding sites may favor the persistence of amphibians by increasing population sizes or number of populations. Also connectivity between breeding sites may increase. However, stressors like diseases and agrochemicals would still be critical. In fact, a dense network of breeding sites could speed up the spreading of diseases. Also, as shown in this thesis, even at distances of less than 1 km between breeding sites, small population sizes and a detectable genetic differentiation of populations can be found in agricultural land. Efforts in the conservation of amphibians should consider stressors in the terrestrial habitat also, especially the exposure to agrochemicals in agricultural land.

In face of the on-going global amphibian declines and alarming predictions of habitat and population loss, researchers and authorities must react quickly and effectively to conserve amphibians in the future.

7 Peer-reviewed research articles

7.1 Pros and cons of external swabbing of amphibians for genetic analyses

Antonia S. Müller & Patrick P. Lenhardt & Kathrin Theissinger (2013)
European Journal of Wildlife Research, 59 (4), 609-612.

7.1.1 Abstract

Non-invasive DNA sampling is an important tool in amphibian conservation. Buccal swabs are nowadays replacing the wounding toe-clipping method. Skin and cloaca swabbing are even less invasive and easier to handle than buccal swabbing, but could result in contaminations of genetic material. Therefore, we test if external skin and cloaca swabs are as reliable as buccal swabs for genetic analysis of amphibians. We analyzed eight microsatellite loci for the common frog (*Rana temporaria*, Linnaeus 1758) and com- pared genotyping results for buccal, skin and cloaca swabs regarding allelic dropouts and false alleles. Furthermore, we compared two DNA extraction methods regarding efficiency and cost. DNA quality and quantity (amplification success, genotyping error rate, in nanogram per microlitre) were comparable among DNA sources and extraction methods. However, skin and cloaca samples exhibited high degrees of contamination with foreign individuals, which was due to sample collection during mating season. Here, we established a simple low budget procedure to receive DNA of amphibians avoiding stressful buccal swabbing or harmful toe clipping. However, the possibility of contaminations of external swabs has to be considered.

7.1.2 Introduction

The global amphibian decline is a well-described problem. Stuart et al. (2004) showed that 43.2%of amphibian species are experiencing some form of population decrease. Apart from climate change, over-exploitation of land and introduction of alien species, the main reasons for this decline are habitat loss and fragmentation (Beebee and Griffiths, 2005; Stuart et al., 2004). For the protection of amphibian populations, information about their genetic structure is necessary to answer questions concerning population size, genetic diversity and gene flow among populations (Frankham et al., 2002). Toe clipping is a common method to collect genetic material of amphibians. However, it often leads to infections and reduction in

mobility (McCarthy and Parris, 2004). To avoid harmful injuries, the buccal swab method was established (Poschadel and Möller, 2004), which is easy to perform, as cheap as other sampling procedures (Pidancier et al., 2003) and resulting in good DNA quality (Broquet et al., 2007). Moreover, storage and transport of swabs is uncomplicated (Poschadel and Möller, 2004), and it is easier to get sampling permission for endangered species (Pidancier et al., 2003). In comparison to toe clipping, buccal swabbing is minimally invasive (Broquet et al., 2007). Nevertheless, opening the mouth of amphibians can be challenging and torturous, especially in small amphibians (e.g. newts). Prunier et al. (2012) already demonstrated the reliability of skin swabs compared to buccal swabs. However, contaminations of external swabs with DNA of foreign individuals are possible especially during mating season or when target species get in close contact during the sampling procedure. Therefore, we tested the reliability of skin and cloaca swabs compared to buccal swabs for genetic analysis of amphibians. Moreover, we compared the effectiveness of two differing DNA extraction methods for swab samples.

7.1.3 Materials and methods

We used as model organism the common frog (*Rana temporaria*) due to an easy handling and microsatellite marker availability. Samples were collected in Germany, Rhineland-Palatinate, near Ramstein-Miesenbach (49.27°N, 7.34°E) during mating season in March 2012. From 20 frogs, three swabs were taken each from buccal mucosa, skin and cloaca (N=60).We used sterile cotton swabs with a wooden handle and separate plastic cover (COPAN, Brescia, Italy). To take the buccal samples, the mouth was opened carefully with a flat plastic tip, and the swab was rolled over the inside of the cheek. For skin sampling, the cotton swab was rolled thoroughly over back, stomach and legs. For cloaca sample, the swab was rolled over the external cloaca. Samples were kept in a cooler box and stored at −23 °C.

Two DNA extraction methods were tested. Extractions of 44 samples (14 buccal, 15 skin, 15 cloaca) were conducted according to high salt DNA extraction protocol after Aljanabi and Martinez (1997) (ca. 0.10 €/sample). DNA of 16 samples (six buccal, five skin, five cloaca) was isolated using the Macherey Nagel NucleoSpin Tissue Kit (Düren, Germany; 2.18 €/sample) following the manufacturer's protocol. Nucleic acid concentrations were measured with NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany).

We analyzed eight variable microsatellite loci (Table 3; Matsuba and Merilä, 2009) and amplified the fragments in two multiplex PCRs by using the QIAGEN Multiplex PCR Kit (Hilden, Germany).

Table 3 Summary data table of eight microsatellite loci. Given are expected (H_E) and observed (H_O) heterozygosity, allelic dropout rate (ADO), false allele rate (FA) and total genotyping error rate (GER=ADO+FA) for buccal, skin and cloaca swabs per locus and over all loci. For buccal swabs, total GER was calculated for both extraction methods additionally

Locus	**BFG066**	**BFG151**	**BFG130**	**BFG082**	**BFG099**	**BFG160**	**BFG145**	**BFG129**	**Over all loci**
Repeat motif	AAG	GAAA	TCTT	TATC	ACTC	TCTA	TCTA	CTAT	
Allele range	143–182	302–394	327–319	207–307	143–195	201–253	154–210	264–628	
Multiplex set	A	A	A	B	B	B	B	B	
H_E/H_O	0.78/0.60	0.87/0.90	0.92/1.00	0.88/0.37	0.78/0.58	0.88/0.79	0.90/0.90	0.88/0.65	0.86/0.72
Buccal swabs									
Number[a]	60	60	60	55	45	56	58	57	
ADO rate (%)	0.0	0.0	1.7	0.0	0.0	1.8	0.0	0.0	0.4
FA rate (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total GER (%)	0.0	0.0	1.7	0.0	0.0	1.8	0.0	0.0	0.4
GS (%)	100.0	100.0	91.5	90.8	72.4	92.7	97.7	96.6	71.8
Skin swabs									
Number[a]	29	27	24	26	16	21	26	29	
ADO rate (%)	0.0	11.1	0.0	0.0	0.0	0.0	0.0	3.4	1.8
FA rate (%)	20.7	7.4	16.7	15.4	0.0	9.5	3.8	10.3	10.5
Total GER (%)	20.7	18.5	16.7	15.4	0.0	9.5	3.8	10.3	12.3
GS (%)	100.0	100.0	91.5	90.8	72.4	92.0	97.7	97.7	41.4
Cloaca swabs									
Number[a]	28	28	25	22	14	21	24	25	
ADO rate (%)	0.0	3.6	0.0	9.1	0.0	0.0	0.0	0.0	1.6
FA rate (%)	25.0	28.6	19.2	4.5	0.0	14.3	8.3	0.0	12.5
Total GER (%)	25.0	32.2	19.2	13.6	0.0	14.3	8.3	0.0	14.1
GS (%)	100.0	100.0	96.4	76.9	57.7	73.1	96.2	100.0	51.9
Total GER (%) for extraction methods									
HSE	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.3
TK	0.0	0.0	0.0	0.0	0.0	7.1	0.0	0.0	0.9

GS percentage genotyping success, *HSE* high salt extraction, *TK* Macherey Nagel NucleoSpin Tissue Kit

[a] Number of successful amplifications

Primers were fluorescently labelled, and 10 pM of primer working solutions were mixed in two multiplex reactions in relation of 1 (Cy5):2 (Cy5.5):4 (Cy5.7). The PCR was conducted in 5 µL of final volume containing 2.5 µL QIAGEN Master Mix, 1.2 µL

QIAGEN Solution, 0.3 µL Primer Mix and 1 µL DNA. PCR was conducted with Primus 96 Cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany) with the following thermal profile: initial denaturation at 95 °C for 15 min, followed by 30 cycles of 30 s at 94 °C, 1.5 min at 55 °C and 1 min at 72 °C. A terminal elongation step was applied for 30 min at 60 °C. Amplification products were run on the CEQ 8000 Sequencer from Beckman Coulter (Krefeld, Germany). Buccal samples were repeated in a multi-tube approach (Taberlet et al., 1996) at least three times to identify real genotypes. Alleles had to be identical across at least two repetitions for heterozygotes and three repetitions for homozygotes (Hansen et al., 2008; Kolodziej et al., 2012). Skin and cloaca samples were repeated up to three times only in case of low amplification success or technical problems.

Fragments were analyzed with the software GeneMarker 1.95 (SoftGenetics, State College, Pennsylvania). The genotypes of the buccal, skin and cloaca swabs of the same individual were compared for consistency, and allelic drop- outs (ADO) as well as false alleles (FA) were counted. Genotyping error rates (GER) were calculated as the sum of ADO and FA for each buccal, skin and cloaca swabs per locus and across all loci. If skin or cloaca genotypes exhibited differing or additional alleles compared to the buccal genotype on at least two loci, we assigned this as a contamination with genetic material of additional individuals, which is also demonstrated by the high number of FAs found in skin and cloaca swabs which seem strongly correlated (Figure 3).

Expected (H_E) and observed (H_O) heterozygosity were calculated with GENETIX 4.04 (http://kimura.univmontp2.fr/genetix/) based on buccal swab results. GER for extraction methods was calculated with buccal swab results as well. Percentage of genotyping success for each swabbing method was calculated over all loci and all repetitions. The raw data table of microsatellite alleles has been uploaded at the dryad database (http://dx.doi.org/10.5061/drayad.4qb53) and can be viewed under link.

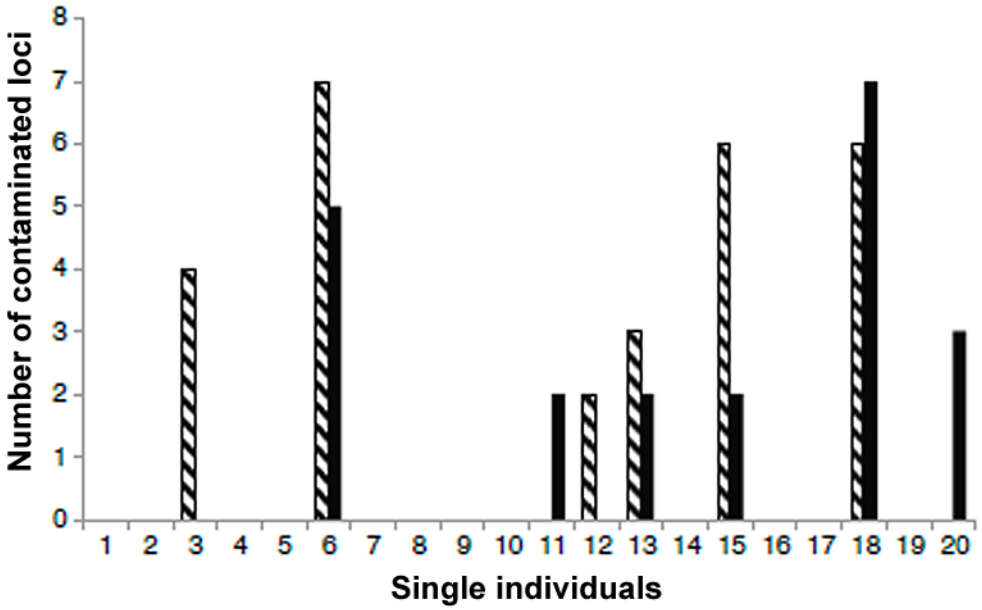

Figure 3 Number of contaminated loci per individual. *Black bars*, cloaca; *striped bars*, skin

7.1.4 Results and discussion

DNA quantity was comparable among both extraction methods. For the high salt extraction procedure, DNA concentrations ranged from 6.0 to 403.2 ng/µL (median=47.6 ng/µL; lower quartile=35.7 ng/µL; upper quartile=76.3 ng/µL). For the commercial kit, DNA concentration ranged from 18.0 to 303.3 ng/µL (median=66.7 ng/µL; lower quartile=58.9 ng/µL; upper quartile=123.0 ng/µL). The high salt DNA extraction resulted in a GER of 0.3%; for the commercial kit extraction, the GER was 0.9% (Table 1). Thus, high salt extraction method is a reliable and cheap alternative to commercial kits. Nucleic acid concentration in DNA extracts of the different swabs ranged from 12.0 to 242.2 ng/µL (median=44.0; upper quartile=63.5; lower quartile=32.9) for buccal swabs; from 8.0 to 207.0 ng/µL (median=51.7; upper quartile=82.5; lower quartile=26.4) for skin swabs; and from 8.1 to 403.0 ng/µL (median=56.5; upper quartile=82.3; lower quartile=44.0) for cloaca swabs. This shows that extracted DNA quantity is comparable among the swab sources.

The genotyping success (GS), i.e. the amplification of each locus, was 71.8% for buccal swabs, 41.4% for skin swabs and 51.9 % for cloaca swabs over all loci, which was mainly due to the non-amplification of one locus (BFG_099). Excluding BFG_099, the GS across the seven remaining loci resulted in 85.1, 62.1 and 72.2%, respectively, which is well in the typical range for microsatellite studies using forensic

samples (GS 20–70%; Kolodziej et al., 2011). For the buccal swabs, the multi-tube approach resulted in a GER of only 0.4% due to ADOs (Table 1). In comparison to other studies using forensic samples, this is a very low GER. Broquet (2004) showed an ADO rate of 11.3% for faeces and 18.7% for hair; in the study of Kolodziej et al. (2013) with faeces of wild boar, a GER of 4.3% occurred. Although faeces and hair samples come along with more potential inhibition problems than buccal swabs, this comparison demonstrates that laboratory and scoring procedures were highly reliable in our study. Compared to the buccal swabs, the skin and cloaca swabs exhibited high GERs of 12.3 and 14.1%, respectively, mainly due to FAs (Table 3). Identical genotypes for all three types of swabs were observed at only 12 of the 20 analyzed individuals (60%). Eight individuals exhibited divergent genotypes at one to seven loci for skin and/or cloaca swabs when compared to the corresponding buccal swab (Figure 3). Our data set partly exhibited the consistency of FAs across skin and cloaca swabs at some loci (N=7), indicating that the high GERs of skin and cloaca swabs was not due to technical problems. The fact that skin and cloaca swabs did not show the same FAs at all instances might be due to the even lower DNA traces of the foreign individuals (e.g. sexual partner), which amplify inconsistently by chance. Moreover, we show the occurrence of four-allele combinations at some loci of skin and cloaca swabs (N=3) (http://dx.doi.org/10.5061/drayad.4qb53), indicating that the high GERs could be attributed to contaminations of target DNA with DNA of a different individual.

This possible contamination could be due to sampling during mating season, when *R. temporaria* is most easily accessible. During the mating procedure, one or several males grasp the females with their forelegs, forming a big lump of frogs. Therefore, the external swabbing of amphibians during mating season, especially for explosive breeders like the common frog or the common toad, could result in high contamination rates. Consequently, skin and cloaca swabs should be applied outside the mating season only. However, this may increase the sampling effort compared to the high and centralized abundance at breeding ponds during the mating season. Cleaning the skin of frogs or toads with water before swabbing may improve the quality of swabs though, also for amphibians caught at fences in buckets. Nevertheless, for newts, e.g. those which are typically caught individually in bottles, skin and cloaca swabs might be feasible.

Beja-pereira et al. (2009) reviewed that non-invasive genetic sampling is nowadays common in wildlife studies and that buccal, skin and cloaca swabs of amphibians and reptiles provide reliable DNA sources for microsatellite analyses. Prunier et al. (2012) also showed that skin swabs are a good alternative to buccal swabs especially for small and vulnerable amphibians. Also for reptiles, cloaca swabs are a reliable alternative to blood sampling and toe clipping (Lanci et al., 2012; Miller, 2006). With this study, we agree that skin, but also cloaca, swabs are easier to conduct, come with a lower risk of injuries for the amphibians and produce comparably good-quality DNA for population genetic analyses. However, we showed that skin and cloaca swabs are not a reliable method of DNA sampling for all amphibian species regarding the respective collection practices. DNA contaminations could lead to distortion of genotyping results what eminently inhibits further analysis. To totally omit the possibility of contaminations with foreign individuals, we recommend that external swabbing should only be conducted if contact of individuals can be excluded.

7.1.5 Acknowledgements

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7.2 Temporal coincidence of amphibian migration and pesticide applications on arable fields in spring

Patrick P. Lenhardt, Carsten A. Brühl & Gert Berger (2015)
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7.2.1 Abstract

Pesticide management may differ between crop species and farm type, particularly with regard to type, number, amount and date of pesticide applications. Such variations in pesticide application strategies entail different temporal coincidence with amphibian species and with species' population proportions. For the first time, we assessed the presence of *Bombina bombina*, *Rana arvalis*, *Pelobates fuscus* and *Triturus cristatus* in agricultural fields. We quantified the temporal coincidence of pesticide applications with the breeding migrations of adult amphibians and evaluated a realistic exposure to pesticides, including the interception by various crops at different growth stages. The level of species-specific coincidence depended on the performed pesticide management, determined by the timing, crop, number and type of pesticide applications. Late migrating species, such as *B. bombina* and *P. fuscus*, overlapped more with pesticide applications than early migrating species, such as *R. arvalis*. Up to 86% of the reproducing population proportion of *P. fuscus* experienced a temporal coincidence with a single pesticide application during stem elongation in winter rape (80% interception). In maize, up to 17% of the reproducing population proportion of *B. bombina* encountered a single herbicide application during bare soil/emergence (no interception). Local monitoring of amphibian migration combined with adjusted pesticide management is recommended to reduce temporal coincidence and thus potential risk of pesticide exposure of amphibians.

7.2.2 Introduction

Intensification of agriculture is discussed as a significant factor for the decline in amphibian populations (Mann et al., 2009; Petersen et al., 2004). With 40% global land coverage, the agricultural landscape is regarded as one of the largest terrestrial biomes on earth (Foley et al., 2005), and also represents an essential habitat for amphibians (Brühl et al., 2013). According to the German Federal Statistical Office (Statistisches Bundesamt 2013), about 52% of German land area was used for agriculture in 2009, 70% of which (about 130,000 km2) was cropped area with associated pesticide applications.

Although the public associates amphibians mostly with an aquatic environment, most temperate amphibians live in terrestrial habitats outside the breeding season for hibernation, foraging and growth. In general, these terrestrial habitats can be kilometers away from breeding ponds (Günther, 1996). In agricultural landscapes, breeding habitats (i.e. ponds and temporary wetlands) often are completely surrounded by arable land. Therefore, amphibians regularly have to cross agricultural land during migration from terrestrial to aquatic habitats for reproduction (Berger et al., 2011; Fryday and Thompson, 2012; Günther and Podloucky, 1996). Exposure to agro chemicals, such as fertilizers and pesticides, is likely during migrations over arable land (Becker et al., 2007; Berger et al., 2012). Due to their permeable skin (Gallant et al., 2007), amphibians are more sensitive than mammals and birds to dermal uptake of chemicals (Quaranta et al., 2009). The dermal uptake of pesticides can cause lethal or sub-lethal effects (Brühl et al., 2011). Uptake via contaminated food is also conceivable, but given the potential for dermal uptake, food intake is less likely to be the major source of pesticide exposure (Smith, 2007). Recent laboratory studies showed high toxicity of commonly used pesticides to terrestrial amphibians at field application rates (Belden et al., 2010; Brühl et al., 2013). Therefore, field cultivation and, in particular, pesticide applications may create a sink for populations. Pesticides may promote local extinctions in combination with reproductive failure (Salice et al., 2011; Taylor et al., 2006) and decreased habitat/population connectivity (Becker et al., 2007; Ficetola and De Bernardi, 2004; Harper et al., 2008).

Whereas the toxicity of pesticides to amphibians is scientifically established (Brühl et al., 2013; Mann et al., 2009), few studies contain detailed information on fine-scale movement patterns in agricultural landscapes (Miaud and Sanuy, 2005; Oromí et al., 2010) or quantify the population proportion affected by applications of agrochemicals (Berger et al., 2012). Such information is often lacking, which makes realistic assessments of terrestrial pesticide exposure of amphibians difficult (Brühl et al., 2011; Smith et al., 2007).

Pesticide management differs between crops and farm, particularly with regard to type, number, amount and date of application of pesticides. Such variations in pesticide application strategies entail different temporal coincidence with amphibian species migrating through or remaining in agricultural fields. Our study was designed to investigate the temporally distinctive spring migration of adult amphibians and its

temporal overlap with pesticide applications. Therefore, we quantified population proportions of four amphibian species migrating just before, during and directly after pesticide applications. We investigated the fire- bellied toad (*Bombina bombina* (Linnaeus, 1761)), moor frog (*Rana arvalis* (Nilsson, 1842)), spadefoot toad (*Pelobates fuscus* (Laurenti, 1768)) and crested newt (*Triturus cristatus* (Laurenti, 1768)), covering a wide range of life cycles and migration types for temperate amphibians. All four species are protected under the European Habitats Directive (EEC 1992). For the first time, we assessed their presence in agricultural fields and obtained a realistic estimate of exposure to pesticides, by also taking into account the interception by various crops at different growth stages. We highlight the critical temporal coincidence of amphibian activity and pesticide applications in order to improve the basis for terrestrial exposure assessment of amphibians.

7.2.3 Materials and methods

Study area

The study area (700 ha) is located 50 km east of Berlin and is part of the young moraine landscape in the northeastern plain of Germany. The climate is continental with an average air temperature of 8.3 °C and a mean annual precipitation of 530 mm (Berger et al., 2011). The landscape mainly consists of arable fields, ranging in size from 10 to 90 ha, and 56 small water bodies ranging from 0.025 to 1.56 ha (median = 0.12 ha). We obtained 331 pesticide application dates from three farms for three crop types and two years: winter cereals (2007: 45 fields; 2008: 55 fields), winter rape (16; 23) and maize (18; 8).

Fence trapping and amphibian migration

The methods to determine the number of captures that recruit into the populations investigated and to ascertain the migration period of amphibians can be found in Berger et al. (2012) and Berger et al. (2013).

Briefly, we installed 26 open, cross-shaped fences in a 400 × 400 m grid within arable land and 23 enclosures sur- rounding ponds and terrestrial habitats like grass edges, hedges, woodlots and set-aside land. We placed pitfall traps at the ends of cross-shaped fences and at 15 m intervals on both sides of the enclosures. Traps were activated in February and then checked daily between 5 a.m. and 10 a.m. until late May in 2007 and 2008. For each catch, we recorded all adult individuals. We

defined the sample population of each species as the total number of trapped migrating adults per migration period. Values ranged from 44 to 1347 trapped migrating adults per period and species. We calculated the relative population proportion migrating per day by using the sample populations and daily counts of trapped adults. Spring migration patterns differed between species with respect to start, end and duration of migration (Figure 4).

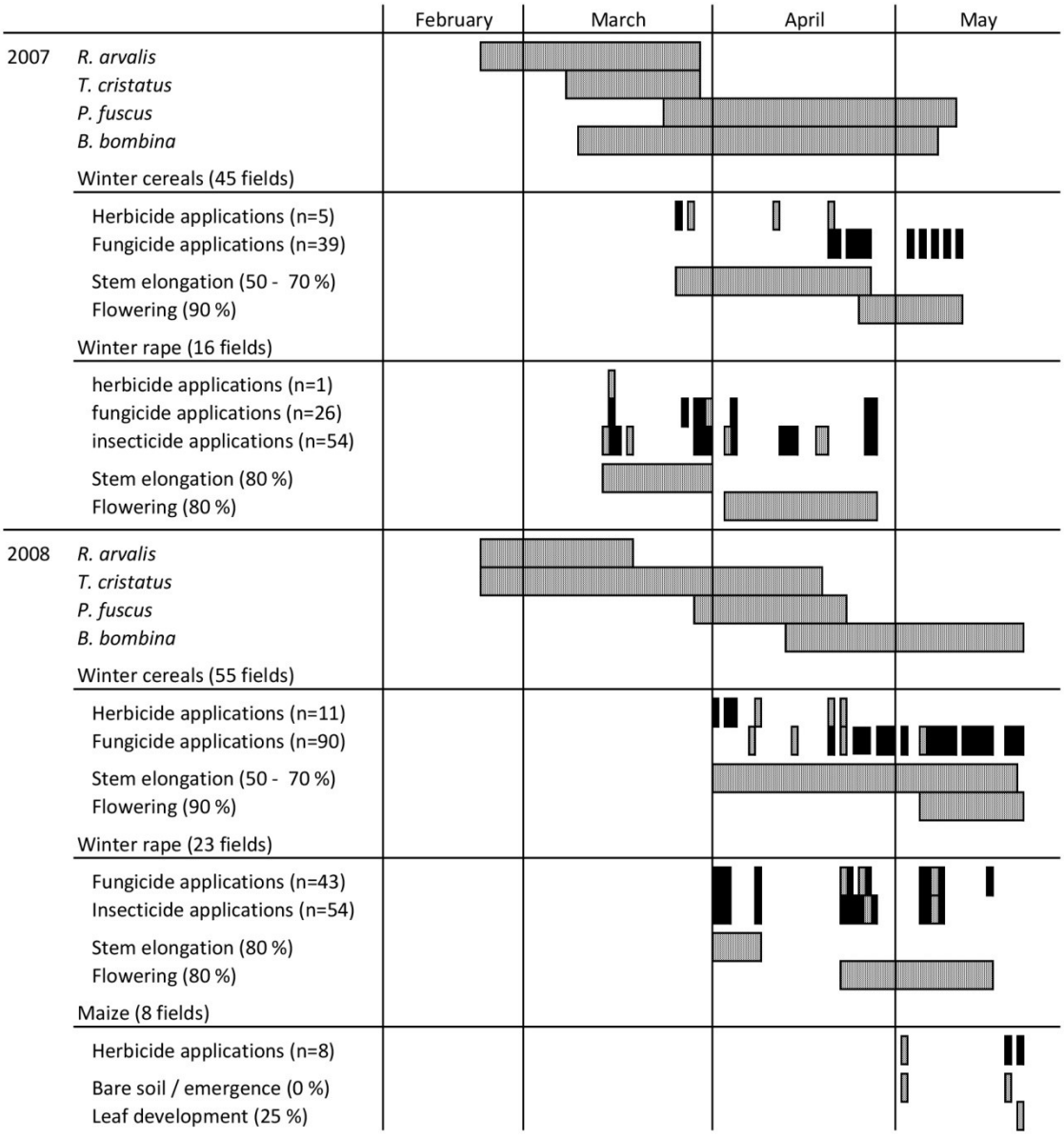

Figure 4 Comparison of adult spring migration and pesticide applications in winter cereals, winter rape and maize. Assumed pesticide interception values in growth stages are given in brackets (according to FOCUS). Days with more than one applied field are marked black.

Up to 25% of the reproducing populations were active on arable land on a single day. However, events with many migrating individuals were rare for all species (Berger et al., 2012).

Differences and similarities in pesticide management

A hierarchical cluster analysis (Ward's method with squared Euclidian distance and Z-value standardization) was performed in SPSS 21 (IBM Corp., 2012) to test for differences and similarities in pesticide management between farms, crops and years (average frequency of active ingredients for insecticides, fungicides and herbicides, and medians of first and last pesticide application grouped by farm, crop and year). Frequencies of applied active ingredients were used instead of frequencies of pesticide applications to account for combinations of multiple pesticides and pesticide formulations with multiple active ingredients. In the following, we will refer to the groups detected in the cluster analyses as pesticide management types (PMTs).

Comparing PMTs with amphibian spring migrations

In order to check for temporal coincidence of amphibian activity during spring migration and pesticide applications, we compared the determined migration periods with the obtained application periods of herbicides, fungicides and insecticides. Because growing stages of the crops were not documented by the farms, we analyzed ontogenetic crop data of the same growing period gained by the crop experimental station at ZALF (Leibniz-Zentrum für Agrarlandschaftsforschung, Germany) located about 5 km from the study area (Roth and Barkusky 2013, personal communication). This allowed us to estimate crop growth stages, as defined by the Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH) (BBCH Working Group, 2001), at the application dates within the study area. For each growth stage, pesticide interception of crops was estimated based on groundwater scenarios of the forum for coordination of pesticide fate models and their use (FOCUS Working Group, 2012).

Assessing temporal coincidence

Temporal coincidence of amphibian presence in arable fields and pesticide application depend on both the time individuals need for passing arable fields and the

persistence of pesticide residues on the soil surface after application. To assess the temporal coincidence, we made the following assumptions:

We took 300 m as an average migration distance from field edges to breeding ponds and assumed an average daily migration distance of 100 m per day for all species (Berger et al., 2012). Thus, we assumed that an average field passage of the individuals takes three days.

After application, pesticides remain on arable fields on plants and soil. We used the half-life (DT_{50}) of the pesticide's active ingredient for vented soil under field conditions to assess the incidence of pesticides after application (Lewis et al., 2013). In the case of applications with multiple active ingredients, the active ingredient with the highest DT_{50} was selected. We assumed an incidence of one day after application for pesticides with a DT_{50} of less than one day. For pesticides with a DT_{50} between one and 50 days, we assumed an incidence of only three days after application. An incidence of 14 days after application was assumed for pesticides with a DT_{50} of more than 50 days.

Finally, we assumed a single pesticide application to be temporally coincident with amphibians present for four (for $DT_{50} < 1$), six (for $DT_{50} \geq 1 \cap \leq 50$) or 17 (for $DT_{50} > 50$) consecutive days in arable fields: (a) three days assumed time for amphibians to pass a field with at least one day of pesticide contact plus (b) one, three or 14 days assumed time of pesticide presence after application.

For each field, we calculated the relative population proportion affected by applications of insecticides, fungicides and herbicides. Multiple applications of one pesticide type on the same field were calculated separately. For each year and species, we calculated the average and maximal relative population proportion affected by applications over all fields of a PMT for all pesticides, and for insecticides, fungicides and herbicides separately. Maximum values represent a worst case scenario in the respective year. We also calculated the average and maximal relative population proportion affected during several crop growth stages, to assess the impact of plant interception as a factor reducing the amount of pesticide to which amphibians are exposed. To this end, we classified all pesticide application records according to crop growth stages (BBCH Working Group 2001) at application date as follows: bare soil/emergence (BBCH 00–09), leaf development (BBCH 10–19), stem elongation (BBCH 20–39), flowering (BBCH 40–89) and senescence/ripening (BBCH 90–99).

7.2.4 Results

Pesticide management types (PMTs)

We analyzed the farm and crop-specific pesticide management from February until late May in 2007 and 2008. Over all farms, 20 fungicide, 14 herbicide and 9 insecticide products were applied with 18, 14 and 8 different active ingredients, respectively. Clustering all pesticide applications revealed a similarity of application patterns within winter rape, maize and winter cereals (winter rye, winter barley, winter wheat and triticale) at all farms and in both years, indicating a rather constant, crop-specific pesticide application pattern (Figure 5). Based on the cluster analyses, we defined the following PMTs: winter rape, maize and winter cereals.

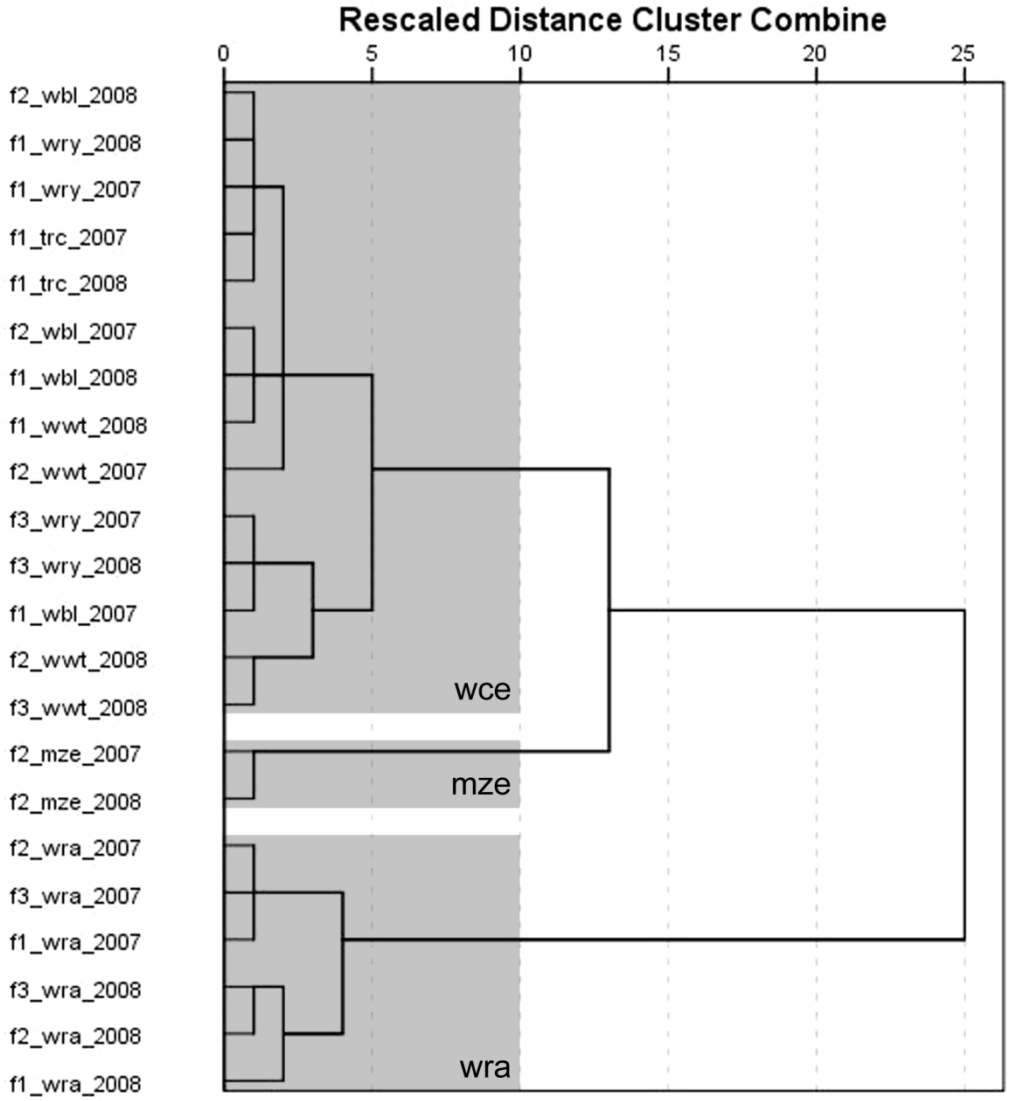

Figure 5 Hierarchical cluster analysis of pesticide applications and frequency (Ward's method) (crops: wry = winter rye, wwt = winter wheat, wbl = winter barley, wce = winter cereals, wra = winter rape, trc = triticale, mze = silage maize; farm number: f1–f3; year: 2007, 2008).

By exploring the crop-specific pesticide application patterns in detail, we noticed a temporal differentiation between crops. One to four fungicide and insecticide applications in winter rape were performed simultaneously in all farms and both years (Figure 4). Furthermore, we detected two delimited application periods for stem elongation and flowering. On winter rape, herbicides were only used by farm 1 in 2007 during stem elongation. As to winter cereals only farm 2 applied insecticides on winter wheat in 2007. Fungicides and herbicides were applied up to two times on winter cereals, but herbicides were only applied during stem elongation.

In general, fungicide applications per crop contained multiple active ingredients with a maximum of seven for winter wheat (farm 1 in 2008) and winter barley (farm 2 in 2007 and farm 1 in 2008). On maize (only cultivated by farm 2), one herbicide application with multiple active ingredients was performed, but fungicide or insecticide was not applied. In 2008, this herbicide application was performed in the middle of May during the last days of spring migration of *B. bombina*.

Overall temporal coincidence and plant interception of pesticides

For both years, *T. cristatus*, *P. fuscus* and *B. bombina* showed temporal coincidence with pesticide applications in winter cereals and winter rape (Figure 4). In 2008, we observed no temporal coincidence for *R. arvalis*, whereas *B. bombina* also showed temporal coincidence with herbicide application in maize.

Across all pesticide types, crop growth stages and species the average population proportion coincident with applications varied between 0.8 and 74.6% (see Appendix A: Figure 6, Figure 7 and Figure 8). On average, more than 20% of the trapped amphibians coincided with each pesticide application in winter cereals (20.7%) and winter rape (22.8%). During stem elongation (BBCH 20–39) a pesticide interception of 50 to 70% (winter cereals) and 80% (winter rape) can be assumed according to FOCUS groundwater scenarios (FOCUS Working Group, 2012). The pesticide interception increases for winter cereals to 90% during flowering (BBCH 40–89), but remains at 80% for winter rape. For temporal coincidence of *B. bombina* with herbicide applications in maize, a pesticide interception of 0% (BBCH 00–09) and 25% (BBCH 10–19) is assumed following FOCUS scenarios (FOCUS Working Group, 2012).

Temporal coincidence in winter cereals

In winter cereals, we observed temporal coincidence of amphibian presence for herbicides and fungicides, but not for insecticides (Figure 4). On average, herbicide applications were temporally coincident with 12.7% (*P. fuscus*) to 36.5% (*T. cristatus*) of the trapped amphibians during stem elongation (50–70% interception) in 2007. Also in 2007, fungicide applications showed only temporal coincidence with the migration of *P. fuscus* (8.4%) and *B. bombina* (8.9%) during stem elongation (Table 1). During flowering (90% interception), migrants of *P. fuscus* (39.6%) and *B. bombina* (0.8%) encountered fungicides applications in 2007, whereas only *B. bombina* (31.6%) encountered fungicides applications in 2008 in winter cereals. With the exception of *R. arvalis* in 2008, we observed temporal coincidence of eleven herbicide applications for all investigated amphibian species during spring migration and stem elongation (50–70% interception) in winter cereals. *P. fuscus* (33.2%) encountered herbicide applications during stem elongation with a maximum population share of 77.9%. In 2008, we observed no temporal coincidence for migrants of *R. arvalis* and fungicide, as well as herbicide applications. The highest proportion of trapped individuals coinciding with one single pesticide application were observed for *P. fuscus* (77.9%; 50–70% interception) and *B. bombina* (59%; 90% interception) in 2008.

Temporal coincidence in winter rape

In winter rape, we observed temporal coincidence of 43 applications and spring migration for all pesticide types and amphibian species during stem elongation (80% interception) in 2007. An unusual herbicide application was performed on winter rape during stem elongation due to unfavorable crop development in 2007, which led to a temporal coincident population proportion between 17.3 and 71.1% (on average 42.2%). In the same year the average temporal coincident population proportion per application during stem elongation (80% interception) varied between 11.6 (*R. arvalis*) and 41.7% (*P. fuscus*) for fungicides and 2.4 to 16.7% (*B. bombina*) for insecticides (Table 4). With the exception of *R. arvalis*, all species showed temporal coincidence with 19 fungicide and 19 insecticide applications during stem elongation (80% interception) of winter rape in 2008. *P. fuscus* and *B. bombina* also showed temporal coincidence with 24 fungicide and 35 insecticide applications during flowering (80% interception).

Temporal coincidence in maize

In 2008, only migrants of *B. bombina* showed temporal coincidence with the herbicide application in maize. During bare soil/emergence (no plant interception), 17% of the sample population were coincident. At the leaf development stage with 25% plant interception, about 12% of the catches were coincident with herbicides on maize fields.

Maximum coincident population proportion

In 2007, the highest relative population share affected by a single herbicide (71.1%) was determined for *T. cristatus* during stem elongation (80% interception) of winter rape (Table 4). Also in 2007, the highest relative population proportion coincident with a single insecticide application (39.8%) was determined for *B. bombina* during flowering (80% interception) of winter rape. In 2008, we observed for *P. fuscus* the highest coincidence values with fungicide and insecticide applications during stem elongation (both 86.6%) in winter rape and by herbicides (77.9%) in winter cereals (50–70% interception).

Table 4 Mean and maximum values of relative population proportions of amphibian species coincident with pesticide applications to field crops in 2007 and 2008 grouped by growth stages (H = herbicides, F = fungicides, I = insecticides, "n. c." = no coincidence, "–" = no application).

		Temporal coincidence of amphibian populations and pesticide applications [%]											
		Stem elongation						Flowering					
		H		F		I		H		F		I	
		mean	max	mean	max	mean	max	mean	max	mean	max	mean	max
Winter cereals													
Interception		50 - 70%						90%					
R. arvalis	2007	14.5	18.3	n. c.	n. c.	-	-	-	-	n. c.	n. c.	-	-
	2008	n. c.	n. c.	n. c.	n. c.	-	-	-	-	n. c.	n. c.	-	-
T. cristatus	2007	36.5	48.6	n. c.	n. c.	-	-	-	-	n. c.	n. c.	-	-
	2008	24.0	33.4	3.0	7.0	-	-	-	-	n. c.	n. c.	-	-
P. fuscus	2007	12.7	17.3	8.4	45.6	-	-	-	-	39.6	45.6	-	-
	2008	33.2	77.9	9.3	47.3	-	-	-	-	n. c.	n. c.	-	-
B. bombina	2007	21.7	32,8	8.9	20.4	-	-	-	-	0.8	2.8	-	-
	2008	10.4	25.0	30.9	53.8	-	-	-	-	31.6	59.0	-	-
Winter rape													
Interception		80%						80%					
R. arvalis	2007	*42.6*	*42.6*	11.6	42.6	13.6	21.4	-	-	n. c.	n. c.	n. c.	n. c.
	2008	-	-	n. c.	n. c.	n. c.	n. c.	-	-	n. c.	n. c.	n. c.	n. c.
T. cristatus	2007	*71.1*	*71.1*	23.7	71.1	14.8	21.4	-	-	n. c.	n. c.	n. c.	n. c.
	2008	-	-	28.6	38.8	26.4	38.8	-	-	n. c.	n. c.	n. c.	n. c.
P. fuscus	2007	*17.3*	*17.3*	23.8	28.8	3.7	4.2	-	-	41.7	45.6	13.0	29.1
	2008	-	-	74.6	86.6	59.6	86.6	-	-	2.3	3.9	3.4	4.5
B. bombina	2007	*37.8*	*37.8*	29.8	40.5	2.4	5.6	-	-	15.2	47.7	16.7	39.8
	2008	-	-	22.8	26.4	21.1	22.9	-	-	21.8	53.8	10.0	16.9
Maize													
		Bare soil / Emergence						Leaf development					
		H		F		I		H		F		I	
		mean	max	mean	max	mean	max	mean	max	mean	max	mean	max
Interception		0%						25%					
R. arvalis	2008	n. c.	n. c.	-	-	-	-	n. c.	n. c.	-	-	-	-
T. cristatus	2008	n. c.	n. c.	-	-	-	-	n. c.	n. c.	-	-	-	-
P. fuscus	2008	n. c.	n. c.	-	-	-	-	n. c.	n. c.	-	-	-	-
B. bombina	2008	17.0	17.7	-	-	-	-	12.0	12.0	-	-	-	-

7.2.5 Discussion

Although some pesticides are known to harm amphibians (Belden et al., 2010; Brühl et al., 2011; Mann et al., 2009; Relyea, 2011; Storrs Méndez et al., 2009), little is known about the coincidences of amphibian activity and pesticide applications (Fryday and Thompson, 2012). The results of our study provide the first assessment of potential temporal coincidences of adult amphibians, moving from hibernation sites into and away from breeding ponds, and applications of multiple pesticide types on arable fields in spring. We assessed the interrelationship of species, crops and pesticide management, to highlight critical coincidence events. Here, we considered pesticide interception by plants as an important factor to assess pesticide exposure in a realistic way (FOCUS Working Group, 2012). Since our study focused on spring migration, the monitored time period from February until late May was appropriate to detect the majority of migrating adults of the investigated species. A more extensive monitoring period should be considered for species that migrate later than the investigated species or when emigration of juveniles from ponds is the main interest.

The migration ability of amphibians depends not only on the species characteristics but also on parameters like soil roughness, density and height of the vegetation cover, health of the migrants, feeding status and environmental conditions during hibernation. We assumed a daily migration rate of 100 m for all species (Berger et al., 2012) and defined groups of the pesticide persistence by applying half-life values of pesticides. These simplifications were made to increase transferability and generality of our results.

We showed that up to 80% of the migrating amphibians temporally coincided with pesticide applications on field during spring migration. As shown by *P. fuscus* in 2008 or *T. cristatus* in 2007, even a single application can lead to high values of temporal coincidence (86.6% and 71.1%, respectively). The temporal coincidence of 17.7% of trapped individuals of *B. bombina* with a single herbicide application in maize due to the extended spring migration in 2008 is rather atypical (in the observed time period), but nonetheless represents a real-world scenario. On average, the temporal coincidences varied between 12 and 41.7% for all species, but depended on PMT. Under favorable circumstances, the majority of early migrants, like *R. arvalis* and *T. cristatus*, may not coincide with the pesticide treatments of arable fields during spring migration (Figure 4). Species active later in the year, like *P. fuscus* and *B. bombina*, are more likely to be exposed to pesticides in fields.

We also showed that for most of the coincident pesticide applications, plant interception in winter cereals and winter rape is high during spring migration. A plant interception of 80% can be assumed for winter rape during stem elongation and flowering. For winter cereals, an interception of 50 to 70% during stem elongation and about 90% during flowering is realistic. Only maize and other summer crops, like sun flowers, provide shelter with an interception rate of 75% during flowering to 90% during senescence/ripening (FOCUS Working Group, 2012). Despite this high interception during migration to the ponds in spring and a wide range of crop cover during emigration from the ponds in summer, amphibians can be harmed by pesticides because 10% of the typical application rate of some pesticides can cause lethal effects (Belden et al., 2010; Brühl et al., 2013). Also the application of pesticides before sowing and during stubble management may be critical for amphibians because vegetation cover of harvested crops is often less than 20% with a high share of bare soil (Berger et al., 2013). In Germany, almost 90% of the glyphosate applications are performed during the pre-sowing and stubble phase (Steinmann et al., 2012).

Regarding the number of pesticides included in this study, we simplified the duration of pesticide presence in the field. This simplification may underestimate the actual temporal coincidence of migrating amphibians and pesticide applications compared with studies investigating only one pesticide (Berger et al., 2013).

Considering amphibian migration during summer and toward hibernation sites prior to winter (Jehle and Arntzen, 2000; Semlitsch, 2008), it is most likely that individuals will encounter multiple applications during an entire year (Berger et al., 2011). Additionally after leaving spawning ponds, species like *P. fuscus* may stay in crop fields that are suitable in terms of soil conditions and food supply (Nöllert and Günther, 1996). Hence, they are under particular risk to be exposed to field cultivation including pesticide application and soil preparation (Berger et al., 2012). This might be indicated by high values of coinciding population proportions (up to about 80%). One major driver of terrestrial activity of amphibians is precipitation or at least higher soil moisture. Favorable air temperature and wet site conditions trigger migration activity of the animals, and may impact both the concentration of chemicals in solutions and uptake via the permeable skin. This may lead to adverse effects.

Aside from contaminated breeding ponds (Bishop et al., 1999; Relyea, 2005), larval development, and hence juvenile emigration, depends on multiple

environmental factors such as predation, desiccation, temperature and food availability. Most juveniles of temperate amphibians emerge from ponds between mid- summer and autumn (Günther, 1996). During this period, a temporal coincidence with pesticide applications is most likely (Berger et al., 2011; Lenhardt et al., 2013). Due to their smaller size and higher sur- face area to volume ratio compared to adults, juveniles could be even more susceptible to toxic effects (Brühl et al., 2011).

It is also likely that migrating amphibians encounter pesticides in combination with natural stressors such as predation, competition, pathogens and climate change (Blaustein et al., 2003; Hof et al., 2011). However, spatial proximity to agriculture may lead to the development of tolerance to some common pesticides, even though circumstances and limitations of the developed pesticide tolerance remain vague (Cothran et al., 2013; Hua et al., 2013). Aquatic invertebrates that adapted to pesticide contamination showed a reduced genetic diversity (Coors et al., 2009). Pesticide tolerance in amphibians may also be correlated with reduced genetic diversity in amphibian populations. A reduced genetic diversity could make amphibians more vulnerable to changing stressors (Lesbarrères et al., 2005). However, it is unclear to which extent pesticide tolerance can be generalized for species and ecosystems (Köhler and Triebskorn, 2013).

7.2.6 Conclusion

Pesticide exposure of amphibians is controlled by three factors: in-field amphibian presence, pesticide application and crop interception. Our results indicate that the presence of amphibians and pesticides in fields overlap regularly. The extent of overlap and interception of pesticides varies between years, crops and amphibian species. As exposure of amphibians to pesticides seems inevitable, we anticipate a certain risk for amphibian populations since mortality can be high at 10% of the field application rate as demonstrated in laboratory experiments.

Based on our findings and due to a substantial lack of knowledge, we recommend more scientific effort to evaluate the potential toxicity of pesticides to ecologically relevant terrestrial amphibian species. We also recommend developing a pesticide risk assessment methodology for amphibians with regard to terrestrial exposure and the dermal pathway in particular. In order to prevent potentially adverse impacts on amphibian populations, the toxicity of pesticides regarding both lethal and sub-lethal effects must be considered. This should include further

investigations on the exposure risk of amphibians to pesticides in terrestrial habitats, crop fields in particular, but should also take the development of risk management strategies into consideration that could consist of adaptations of pesticide applications during amphibian migration.

7.2.7 Acknowledgements

Field work of this study was supported by the German Environmental Foundation (DBU). Additional technical staff conducted the practical work, farmers provided valuable data on their field management, R. Roth and D. Barkusky provided data on the growth stages of crops. The authors thank all contributing parties for their participation.

7.2.8 Appendix A

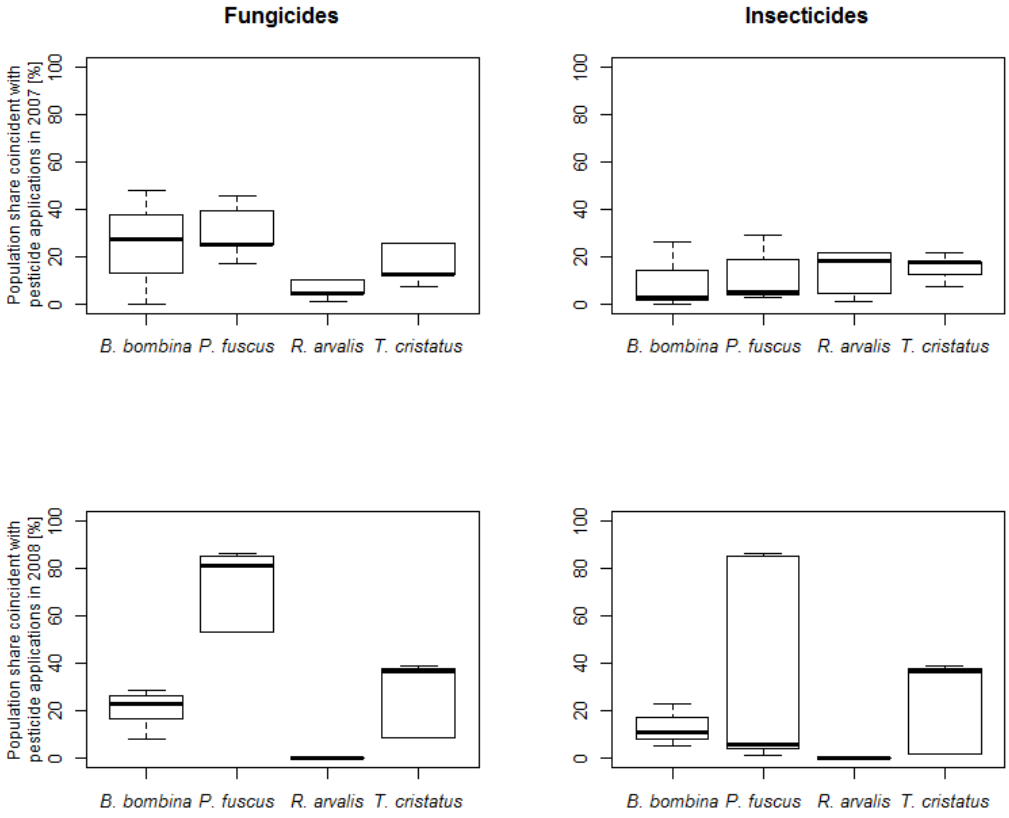

Figure 6 Boxplots of temporal coincidence of amphibian species with pesticide applications in winter rape during spring migration 2007 and 2008 (median values and boxes with 25 and 75% quantiles; error lines indicate lowest and highest values).

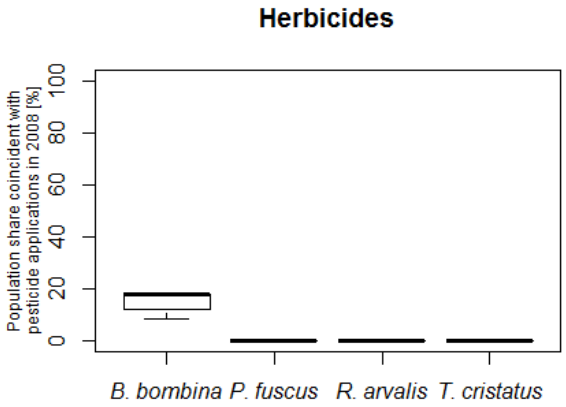

Figure 7 Boxplots of temporal coincidence of *B. bombina* with herbicide applications in maize during spring migration 2007 and 2008 (median values and boxes with 25 and 75% quantiles; error lines indicate lowest and highest values).

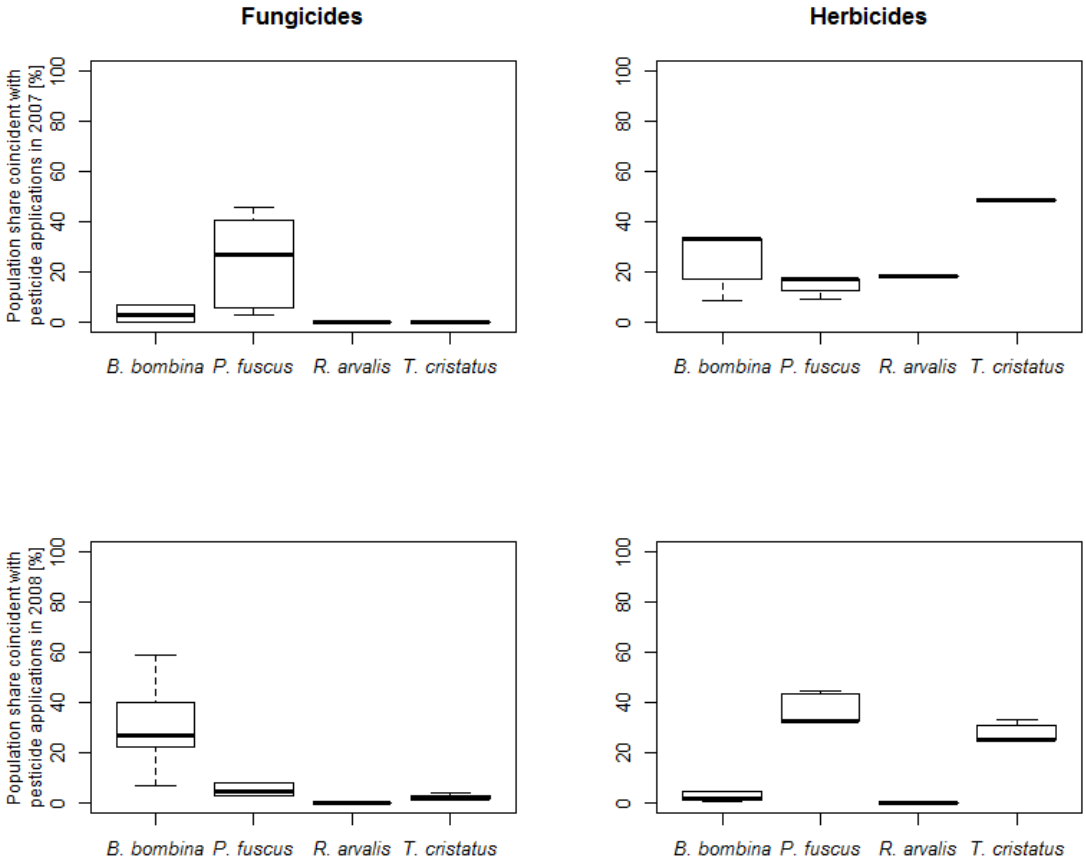

Figure 8 Boxplots of temporal coincidence of amphibian species with pesticide applications in winter cereals during spring migration 2007 and 2008 (median values and boxes with 25 and 75% quantiles; error lines indicate lowest and highest values).

Table 5 List of the recorded pesticides applied in the study area.

	pesticide	pesticide type
winter rape	Agil-S	herbicide
	Biscaya	insecticide
	Bulldock	insecticide
	Cantus	fungicide
	Caramba	fungicide
	Fastac SC Super Contact	insecticide
	Folicur	fungicide
	FURY 10 EW	insecticide
	Priori Xtra	fungicide
	Proline	fungicide
	Reldan 22	insecticide
	Talstar 8 SC	insecticide
	Trafo WG	insecticide
	Ultracid 40	insecticide
maize	Callisto	herbicide
	Glyfos	herbicide
	Motivell	herbicide
	Primagram Gold	herbicide
winter cereals	Alto 240 EC	fungicide
	Amistar	fungicide
	Arelon (fluid)	herbicide
	Axial	herbicide
	Azur	herbicide
	Capalo	fungicide
	Champion	fungicide
	Cirkon	fungicide
	CONCERT SX	herbicide
	Corbel	fungicide
	Diamant	fungicide
	Fandango	fungicide
	Folicur	fungicide
	Gladio	fungicide
	Input	fungicide
	MCPA Berghoff	herbicide
	Monitor	herbicide
	Opus Top	fungicide
	Platform S	herbicide
	Pointer SX	herbicide
	Primus	herbicide
	Priori Xtra	fungicide
	Pronto PLUS	fungicide
	Vegas	fungicide

7.3 Repeated randomized selection of genotypes for reliable estimates of population differentiation in data containing siblings

Patrick P. Lenhardt & Kathrin Theissinger (2017)
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7.3.1 Abstract

In population genetic studies a proper sampling design is crucial for reliable population differentiation estimates. For genetic studies on amphibians, fish or insects, larvae are often sampled instead of adults due to their higher accessibility and abundance. However, population genetic parameters derived from larval (sibling) sampling can be biased if adults are represented unevenly in the larval population. The removal of full-siblings from data may improve the quality of the results but entails in a low number of individuals per site, especially in small populations. Using simulated data of ten microsatellite loci for ten populations, we estimated pairwise F_{ST} and R_{ST} values as well as F_{IS} and R_{IS} values for the F0 generation (parental) and the F1 generation (descendant). We applied a repeated randomized selection of genotypes (RRSG) to investigate the impact of removing full-siblings from the data. We also investigated the impact of reduced sample size on the results generated by RRSG as well as the advantages of RRSG over a single estimate of genetic parameters after removal of full-siblings. The RRSG approach produced pairwise F_{ST} values that deviated on average only by 0.0050 (N=45) from their respective estimates for F0. Estimates for H_E deviated less the 5% over all data sets. We henceforth suggest applying the described method to interpretations of genetic differentiations in studies with i) small effective population sizes and ii) data containing siblings where iii) offspring can be assigned to at least one parent due to reproduction practice.

7.3.2 Introduction

Landscape genetic studies give important insights in dynamics and structure of natural populations. Estimating the amount of genetic differentiation among populations is hereby a major goal to understand the demographic development of populations. The majority of landscape genetic studies apply microsatellites (Storfer et al., 2010), since such variable loci are well suited for fine-scale genetic analysis (Cavers et al., 2005). F_{ST} (Wright, 1951) and R_{ST} (Slatkin, 1995) are common measures to estimate the degree of genetic differentiation among groups of

individuals. The reliability of F_{ST} and R_{ST} can be improved by increasing the number of loci and samples (Morin et al., 2009; Willing et al., 2012). Increasing the set of loci can be crucial when genetic studies are based on declining or small populations. Yet, the number of suitable markers may be limited for non-model species. On the other hand, increased sample sizes (individuals per population) promote reliable population differentiation estimates (Kalinowski, 2005; Selkoe and Toonen, 2006) while small sample sizes may cause an over- or underestimation of genetic parameters (Willing et al., 2012). Therefore, most genetic studies on wild populations aim to use more than 20 individuals per population to estimate genetic differentiation (Willing et al., 2012). However, in wildlife research it is not always achievable to obtain such high samples sizes, due to small populations, limited time period for sampling (e. g. when sampling is only feasible during breeding phase) and limitations through authorities (e. g. animal welfare regulations).

In population genetic studies on amphibians (e.g., Ficetola et al., 2008; Morgan et al., 2008), fishes (e.g., Hansen et al. 1997) or insects (e.g., Taubmann et al. 2011) larvae are often sampled instead of adults due to their higher accessibility and abundance. Yet, larvae represent only a proportion of the total effective population since not every adult may breed every season (Niemelä et al., 2006) or has mating success. When adults are represented unevenly in the larval population, the removal of siblings may correct for the biased contribution of adults (Goldberg and Waits, 2010), which may again result in small sample sizes ($n < 20$).

In this study, we investigate a novel method to obtain reliable estimates of population differentiation with small population sizes by utilizing simulated microsatellite data of siblings in a repeated randomized selection of genotypes (RRSG) approach. We estimated observed (H_O) and (H_E) expected heterozygosity, pairwise F_{ST} and R_{ST} values as well as F_{IS} and R_{IS} values for the F0 generation and the F1 generation, with and without full-siblings in the data set. We reduced the number of parent couples to test for sample size related effects on the estimated genetic parameters. Finally, we compared our results with the estimations based on data from the F0 generation and data containing siblings.

7.3.3 Materials and Methods

We used EASYPOP v 2.0.1 (Balloux, 2001) to simulate a meta-population of diploid individuals for ten populations (P1 to P10, with 20 females and 20 males each), considering ten unlinked loci and a mutation rate of 0.0004 using the single step

mutation model. We chose the island model as migration model and assumed a migration rate of 10%. We created 20 couples per population (parental generation, F0) and simulated three offspring (full-siblings) per couple (descendant generation, F1-20sib, N = 60 per population).

We calculated Hardy-Weinberg-Equilibrium (HWE), linkage disequilibrium (LD), H_O and H_E, pairwise F_{ST} and R_{ST} as well as F_{IS} and R_{IS} values for the F0 and F1-20sib data sets with GenePop on the web 4.2 (Raymond and Rousset, 2004; Rousset, 2008). To remove bias from F1-20sib due to siblings in the data, it is current practice to randomly select one offspring per couple for genetic analysis (single randomized selection of genotypes). However, since estimated genetic parameters may vary due to the selection of one sibling over another, we performed this step not only once but 200,000 times by randomly selecting one offspring per couple (repeated randomized selection of genotypes; RRSG), resulting in 200.000 data subsets with 20 individuals per population (F1-20). To test for effects of sample size, we repeated the RRSG approach with a reduced number of parental couples for the data sets F1-19 (19 parental couples) to F1-7 (seven parental couples).

We calculated H_O and $H_{E,}$ pairwise F_{ST} and R_{ST} as well as F_{IS} and R_{IS} values by automatically executing all data subsets with GenePop batch (Rousset, 2008). For F1-7 to F1-20 we verified normal distribution of the H_O and $H_{E,}$ pairwise F_{ST} and R_{ST} as well as F_{IS} and R_{IS} values with histograms and QQ-Plots using R (Figure 9).

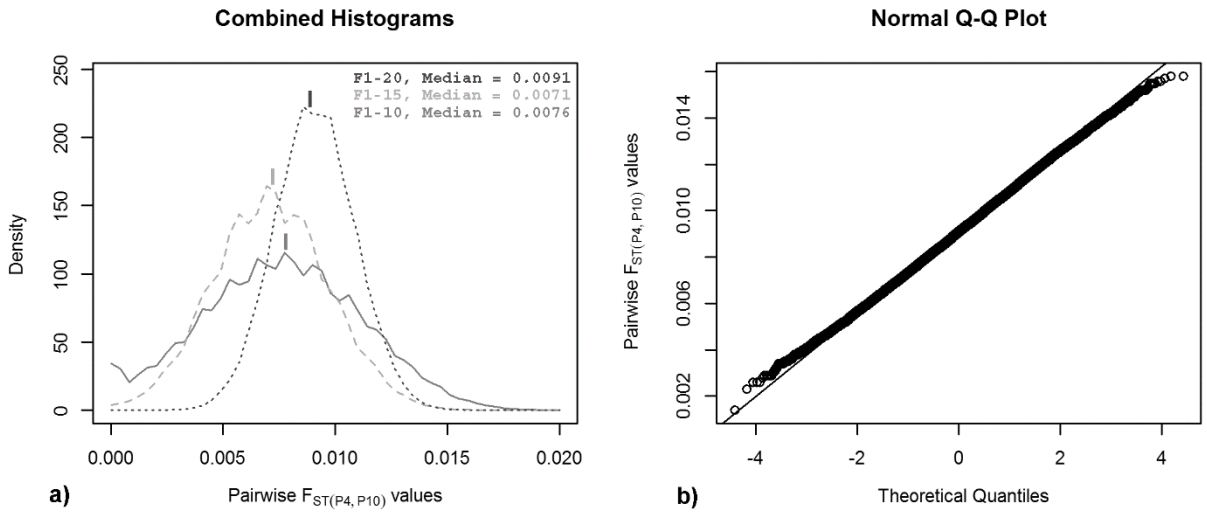

Figure 9 Exemplary presentation of the pairwise F_{ST} results of the RRSG approach for the population pair P4, P10 for the data sets F1-20, F1-15 and F1-10 (a) and the according QQ-Plot (b).

Although all original data was normally distributed, we chose median over mean values, since we lost normal distribution in some cases due to the replacement of negative values with zero. Moreover, medians are more robust against outliners (Eichler and Vogel, 2011).

For F1-7 to F1-20, we compared median pairwise F_{ST} values of 10.000 to 200.000 subsets in steps of 10.000 to determine an adequate number of calculations for reliable median H_O and H_E, median pairwise F_{ST} and R_{ST} as well as F_{IS} and R_{IS} values, which was assumed if the median pairwise F_{ST} values differed less than 0.0003 between all population pairs when additional 10.000 subsets were considered.

We compared the overall genetic estimates between F0, F1-20sib and F1-20 to F1-7. Afterwards we tested median H_O and H_E, median pairwise F_{ST} and R_{ST} as well as F_{IS} and R_{IS} values of F0, F1-20sib and F1-20 for significant differences with the Mann-Whitney-U-Test (MWU). We compared minimum, maximum and median of H_O and H_E, pairwise F_{ST} and R_{ST} as well as F_{IS} and R_{IS} values from F1-7 to F1-20 to test for possible sampling size effects.

7.3.4 Results and Discussion

All simulated loci were polymorphic with a range of 49 to 61 alleles per locus. Allele sizes covered a wide range (002 – 095) and were evenly distributed across the simulated data set. For F0, no population showed heterozygote excess and over all populations no LD was detected, whereas F1-20sib showed heterozygote excess in four out of ten simulated subpopulations (P3, P4, P5 and P10).

Overall, F1-20 showed higher levels of population differentiation (pairwise F_{ST} and R_{ST}) than F0, but F1-20 values were not outside the 95% confidence interval (CI) of the F0 estimates. Global median estimates of pairwise F_{ST} and R_{ST} as well as F_{IS} in F1-20 were closer to the estimates of F0 than estimates of F1-20sib, indicating potentially higher bias due to siblings in the data set. Only for global R_{IS}, the estimate of F1-20sib was closer to F0 than the median estimate of F1-20. Especially for global F_{ST}, the median estimates of F1-20 to F1-7 were close to the estimated F_{ST} in F0 (Table 6). Also for global R_{ST}, median estimates of F1-20 to F1-7 (with exception of F1-9) were closer to F0 than the estimate of F1-20sib. For global F_{IS} and R_{IS} only median estimates of F1-20 to about F1-14 were within close range to estimated values of F0, although median estimates down to F1-7 were within the 95% CI of F0.

When comparing respective pairwise F_{ST} values of F0, F1-20sib and F1-7 to F1-20, estimates of F1-20sib deviated on average by 0.0089 (min: 0.0003 – max: 0.0142) from F0, whereas median estimates of F1-20 only deviated by 0.0027 (0.0000 – 0.0070). Even in F1-7, pairwise F_{ST} values only deviated by 0.0052 (0.0001 – 0.0130) on average from their respective values in F0. MWU-Test showed statistically significant differences of F1-20sib to F0 and F1-20 to F1-7 ($p = <0.0001$), whereas F1-20 to F1-7 were not statistically significant different from F0. The reduction of sample size resulted in higher estimates of population differentiation. However, since F1-7 was still in the 95% CI of F0 and not statistically significant different from F0, we consider these higher estimates within reason.

When comparing respective pairwise R_{ST} values of F0, F1-20sib and F1-20, estimates of F1-20sib deviated on average by 0.0105 (0.0000 – 0.0365) from F0, whereas median estimates of F1-20 only deviated by 0.0052 (0.0000 – 0.0344). MWU-Test showed statistically significant differences of F1-20sib to F0 and F1-20 to F1-14 ($p = <0.0010$), whereas F1-20 to F1-18 were not statistically significant different from F0.

When comparing respective F_{IS} values of F0 and F-20 to F1-7, estimates of F1-20 to F1-12 deviated by about 0.0130 (0.0000 – 0.0280) on average from F0, whereas for F1-11 to F1-7 deviation increased up to about 0.0160 (0.0000 – 0.0358). F_{IS} values of F1-20sib and F1-20 were both not statistically significant different from F0.

Table 6 Global values of F_{ST}, R_{ST}, F_{IS} and R_{IS} and their respective 95% confidence intervals for F0 (parental generation), F1-20sib (descendant generation with siblings). Estimates of F1-7 (seven parental couples, three offspring per couple) to F1-20 (twenty parental couples, three offspring per couple) were calculated using the repeated randomized selection of genotypes approach.

Data	Global F_{ST}	95% CI	Global R_{ST}	95% CI	Global F_{IS}	95% CI	Global R_{IS}	95% CI
F0	0.0077	0.0015 - 0.0162	0.0000	0.0000 - 0.0208	0.0031	0.0000 - 0.0265	0.0302	0.0000 - 0.1245
F1-20sib	0.0165	0.0120 - 0.0205	0.0117	0.0000 - 0.0343	0.0000	0.0000 - 0.0102	0.0329	0.0000 - 0.0881
F1-20	0.0079	0.0028 - 0.0120	0.0050	0.0000 - 0.0306	0.0030	0.0000 - 0.0200	0.0401	0.0000 - 0.1044
F1-19	0.0080	0.0026 - 0.0122	0.0059	0.0000 - 0.0304	0.0026	0.0000 - 0.0185	0.0306	0.0000 - 0.0936
F1-18	0.0085	0.0032 - 0.0129	0.0071	0.0000 - 0.0296	0.0001	0.0000 - 0.0190	0.0236	0.0000 - 0.0747
F1-17	0.0087	0.0034 - 0.0135	0.0063	0.0000 - 0.0299	0.0025	0.0000 - 0.0218	0.0300	0.0000 - 0.0935
F1-16	0.0083	0.0025 - 0.0135	0.0054	0.0000 - 0.0296	0.0027	0.0000 - 0.0261	0.0232	0.0000 - 0.1009
F1-15	0.0082	0.0014 - 0.0135	0.0053	0.0000 - 0.0312	0.0033	0.0000 - 0.0259	0.0221	0.0000 - 0.0886
F1-14	0.0083	0.0010 - 0.0159	0.0037	0.0000 - 0.0339	0.0025	0.0000 - 0.0257	0.0316	0.0000 - 0.0894
F1-13	0.0086	0.0021 - 0.0155	0.0044	0.0000 - 0.0374	0.0055	0.0000 - 0.0177	0.0131	0.0000 - 0.1061
F1-12	0.0088	0.0018 - 0.0155	0.0055	0.0000 - 0.0395	0.0049	0.0000 - 0.0200	0.0153	0.0000 - 0.0880
F1-11	0.0091	0.0012 - 0.0169	0.0067	0.0000 - 0.0485	0.0072	0.0000 - 0.0275	0.0112	0.0000 - 0.1327
F1-10	0.0090	0.0000 - 0.0155	0.0069	0.0000 - 0.0496	0.0084	0.0000 - 0.0239	0.0144	0.0000 - 0.1306
F1-9	0.0086	0.0000 - 0.0177	0.0117	0.0000 - 0.0601	0.0087	0.0000 - 0.0217	0.0322	0.0000 - 0.1079
F1-8	0.0090	0.0000 - 0.0184	0.0097	0.0000 - 0.0604	0.0117	0.0000 - 0.0279	0.0359	0.0000 - 0.1040
F1-7	0.0086	0.0000 - 0.0186	0.0114	0.0000 - 0.0796	0.0144	0.0000 - 0.0345	0.0123	0.0000 - 0.1471

Yet, F1-20sib was statistically significantly different from F1-20 to F1-7 (p = <0.0400). For R_{IS}, we detected no statistically relevant difference between data sets. Overall, R_{IS} in F1-20 to F1-7 deviated by 0.0500 from results of F0. Median H_O and H_E values over all loci showed no statistically significant difference between F0 and F1 data sets for all populations (7.3.5 Appendix A Table 7).

We also applied the RRSG approach to a real data set with three populations and ten loci (5 to 31 alleles per locus) and an H_E of 0.743. Analogous to the results based on the simulated data, we observed stable median values for H_E /H_O and pairwise F_{ST} and R_{ST} as well as F_{IS} and R_{IS} (data not shown).

The RRSG approach clearly produced more reliable estimates of pairwise F_{ST} values than the F1-20sib data set. Compared to the simple removal of siblings from data, RRSG also has the advantage of returning a range of values and not just a single value that might strongly depend on which genotypes were selected (Figure 9a).

Overall, pairwise F_{ST} performed better than pairwise R_{ST} for our simulated data. The RRSG approach produced improved estimates of pairwise R_{ST} compared to F1-20sib. However, estimates generated with RRSG became less reliable when sample size was reduced. The better RRSG performance of the pairwise F_{ST} values compared to the pairwise R_{ST} values could be due to the underlying mutation model. Since the simulated data showed a wide range of evenly distributed allele sizes (002 – 095), the random selection of some alleles should result in more severe changes under the stepwise mutation model (R_{ST}) as opposed to the infinite allele model (F_{ST}). In real data sets, where allele ranges might not be so wide and alleles not so evenly distributed, the difference between F_{ST} and R_{ST} might not be so prominent anymore.

Since related individuals are more likely to share identical genotypes than unrelated individuals (Taberlet and Luikart, 1999), the removal of full-siblings from larvae samples improves estimates of population and landscape genetic parameters (Goldberg and Waits, 2010). Therefore, the removal of full-siblings gets widely applied in numerous studies (Olafsson et al., 2014; Peterman et al., 2015; Trumbo et al., 2013; Wang, 2012). Some authors even claim that forming multiple random subsets for estimates of genetic parameters would not be necessary after the removal of full siblings, since similar estimates could be expected (Whiteley et al., 2014). Yet, we were able to show that forming multiple random subsets can in some cases produce a wide range of genetic parameters estimates. Also, the removal of

full-siblings may result in a very small sample size, not suitable for statistical analyses with current methods. For all of the investigated genetic estimates, we observed a wider range between minimum and maximum values when sample size was reduced, whereas median values remained stable. This indicates that a result from a single randomized selection of genotypes may be an outlier and does not reflect the real underlying genetic signal.

Our approach for removing full-siblings from a simulated offspring data set using RRSG produced pairwise F_{ST} estimates which were closer to estimates calculated for the simulated parental data set, compared to estimates based on data containing siblings. RRSG still produced pairwise F_{ST} estimates close to those calculated for F0 when sample size (number of parental couples) was reduced. Any potential bias due to selection of one sibling over another was compensated by performing multiple estimates of the genetic parameters. We find our results based on RRSG from F1 generations sufficient for reliable population differentiation estimates, without the requirement of a large sample size. We henceforth suggest applying the described method and using median pairwise F_{ST} values for further interpretations of population genetic data in studies with i) small effective population sizes and ii) data containing siblings where iii) offspring can be assigned to at least one parent due to reproduction practice. A beta version of the Windows software used to automatically generating and executing input files with repeated randomized selection of genotypes can be requested from the authors.

7.3.5 Appendix A

Table 7 Expected (H_E) and observed (H_O) heterozygosity over all loci for populations P1 to P10 for data sets F0, F1-20sib and F1-20 to F1-7.

	P1		P2		P3		P4		P5	
Data set	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O
F0	0.967	0.970	0.969	0.960	0.965	0.940	0.954	0.930	0.961	0.950
F1-20sib	0.955	0.943	0.959	0.965	0.952	0.968	0.946	0.950	0.952	0.963
F1-20	0.963	0.945	0.966	0.965	0.960	0.970	0.954	0.950	0.961	0.965
F1-19	0.963	0.947	0.966	0.963	0.959	0.968	0.953	0.953	0.961	0.968
F1-18	0.963	0.944	0.965	0.967	0.959	0.967	0.952	0.956	0.960	0.967
F1-17	0.962	0.941	0.966	0.965	0.959	0.971	0.953	0.953	0.960	0.965
F1-16	0.964	0.938	0.967	0.956	0.959	0.969	0.954	0.950	0.960	0.969
F1-15	0.965	0.940	0.967	0.960	0.959	0.967	0.951	0.947	0.960	0.973
F1-14	0.967	0.943	0.969	0.964	0.959	0.971	0.953	0.950	0.960	0.964
F1-13	0.967	0.946	0.969	0.962	0.958	0.969	0.954	0.954	0.959	0.969
F1-12	0.967	0.950	0.969	0.967	0.960	0.967	0.952	0.950	0.960	0.975
F1-11	0.967	0.945	0.969	0.955	0.956	0.964	0.952	0.955	0.961	0.982
F1-10	0.967	0.950	0.971	0.960	0.955	0.960	0.952	0.950	0.961	0.980
F1-9	0.967	0.956	0.970	0.956	0.957	0.967	0.950	0.944	0.961	0.978
F1-8	0.967	0.950	0.969	0.950	0.955	0.963	0.948	0.938	0.961	0.975
F1-7	0.966	0.929	0.966	0.943	0.952	0.957	0.946	0.929	0.965	0.986
	P6		**P7**		**P8**		**P9**		**P10**	
Data set	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O
F0	0.952	0.955	0.958	0.960	0.959	0.960	0.962	0.965	0.962	0.955
F1-20sib	0.953	0.955	0.949	0.947	0.953	0.958	0.952	0.955	0.955	0.972
F1-20	0.960	0.950	0.957	0.940	0.959	0.960	0.960	0.955	0.962	0.970
F1-19	0.959	0.947	0.957	0.942	0.959	0.958	0.960	0.958	0.963	0.968
F1-18	0.959	0.956	0.955	0.939	0.958	0.956	0.960	0.961	0.964	0.972
F1-17	0.958	0.953	0.957	0.935	0.957	0.953	0.959	0.959	0.964	0.971
F1-16	0.956	0.950	0.956	0.938	0.957	0.956	0.959	0.963	0.964	0.969
F1-15	0.957	0.953	0.957	0.933	0.958	0.953	0.959	0.953	0.963	0.967
F1-14	0.956	0.950	0.955	0.929	0.960	0.950	0.958	0.957	0.963	0.964
F1-13	0.954	0.946	0.959	0.946	0.958	0.954	0.961	0.954	0.963	0.962
F1-12	0.954	0.950	0.961	0.942	0.959	0.950	0.956	0.950	0.961	0.958
F1-11	0.952	0.945	0.963	0.936	0.959	0.945	0.956	0.945	0.962	0.964
F1-10	0.951	0.950	0.966	0.950	0.959	0.940	0.958	0.950	0.958	0.960
F1-9	0.960	0.956	0.969	0.956	0.959	0.944	0.954	0.944	0.959	0.956
F1-8	0.959	0.950	0.969	0.950	0.958	0.938	0.957	0.950	0.960	0.950
F1-7	0.962	0.971	0.968	0.943	0.959	0.929	0.958	0.943	0.964	0.957

7.4 Amphibian population genetics in agricultural landscapes: does viniculture drive the population structuring of the European common frog (*Rana temporaria*)?

Patrick P. Lenhardt, Carsten A. Brühl, Christoph Leeb & Kathrin Theissinger (2017)
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7.4.1 Abstract

Amphibian populations have been declining globally over the past decades. The intensification of agriculture, habitat loss, fragmentation of populations and toxic substances in the environment are considered as driving factors for this decline. Today, about 50% of the area of Germany is used for agriculture and is inhabited by a diverse variety of 20 amphibian species. Of these, 19 are exhibiting declining populations. Due to the protection status of native amphibian species, it is important to evaluate the effect of land use and associated stressors (such as road mortality and pesticide toxicity) on the genetic population structure of amphibians in agricultural landscapes. We investigated the effects of viniculture on the genetic differentiation of European common frog (*Rana temporaria*) populations in Southern Palatinate (Germany). We analyzed microsatellite data of ten loci from ten breeding pond populations located within viniculture landscape and in the adjacent forest block and compared these results with a previously developed landscape permeability model. We tested for significant correlation of genetic population differentiation and landscape elements, including land use as well as roads and their associated traffic intensity, to explain the genetic structure in the study area. Genetic differentiation among forest populations was significantly lower (median pairwise F_{ST} = 0.0041 at 5.39 km to 0.0159 at 9.40 km distance) than between viniculture populations (median pairwise F_{ST} = 0.0215 at 2.34 km to 0.0987 at 2.39 km distance). Our analyses rejected isolation by distance based on roads and associated traffic intensity as the sole explanation of the genetic differentiation and suggest that the viniculture landscape has to be considered as a limiting barrier for *R. temporaria* migration, partially confirming the isolation of breeding ponds predicted by the landscape permeability model. Therefore, arable land may act as a sink habitat, inhibiting genetic exchange and causing genetic differentiation of pond populations in agricultural areas. In viniculture, pesticides could be a driving factor for the observed genetic impoverishment, since pesticides are more frequently applied than any other

management measure and can be highly toxic for terrestrial life stages of amphibians.

7.4.2 Introduction

The survival of amphibian wildlife populations is threatened by habitat loss, fragmentation of populations, diseases, invasive species, climate change and toxic substances (Stuart et al., 2008). Underlying causes of habitat loss, fragmentation and habitat pollution with toxic substances are the expansion and intensification of agriculture (Gallant et al., 2007; Hartel et al., 2010) as well as built-up areas due to the development of traffic infrastructure, urbanization and industrialization (Löfvenhaft et al., 2004). While the hazard of built-up areas for amphibians is obvious (i.e., roads with car traffic as physical barriers), the threat of agriculture is more complex. Beside habitat loss and fragmentation of remaining suitable habitats or populations, agriculture often requires the development of irrigation, drainage and/or retention systems, which can impact the availability and quality of amphibian breeding sites. Yet despite their limited dispersal capacity compared with other vertebrates (Hillman et al., 2014), amphibians have been able to persist in agricultural landscapes by adapting to the altered availability of breeding sites (Mann et al., 2009). In agricultural landscapes, breeding habitats are often completely surrounded by arable land (Berger et al., 2011). Thus, amphibians regularly have to cross agricultural land during dispersal and seasonal migration (i.e., spring migration for reproduction) or for foraging and are therefore likely exposed to field cultivation measures (Becker et al., 2007; Joseph Mitchell, 2016; Lenhardt et al., 2015).

The expansion and intensification of agriculture also involves input of a wide variety of agrochemicals into the environment. Pesticides play a crucial role in this context, since they can be highly toxic to terrestrial life stages of amphibians (Brühl et al., 2013; Cusaac et al., 2016). Additionally, a spatio-temporal overlap of pesticide applications with the terrestrial activity phase of amphibians was demonstrated for some crops (Lenhardt et al., 2015). In a terrestrial exposure scenario, application-relevant rates of fungicides caused mortality rates of approximately 70% (Belden et al., 2010) and 100% (Brühl et al., 2013) of amphibian test organisms. Also, the use of two or more pesticides in a mixture application is very common and may cause higher toxicity compared to non-mixture applications (Brodeur et al., 2014; Kumar, 2014). Furthermore, pesticides from different applications may accumulate in surface waters (Ulrich et al., 2015), exposing adult amphibians and their larvae to a diverse

pesticide mixture. The demonstrated sublethal and lethal toxicity of various pesticides on aquatic and terrestrial life stages of amphibians (Denoël et al., 2013; Ghose et al., 2014; Lau et al., 2015; Relyea, 2011; Sparling and Fellers, 2009) suggests a potentially strong selection effect on meta-populations in agricultural landscapes. Furthermore, mortality or reduced locomotion capacity of amphibians due to pesticide exposure may promote the fragmentation of breeding pond populations (Lenhardt et al., 2013).

An indirect method to assess the effect of fragmentation on amphibian breeding pond populations is the use of neutral molecular markers, such as polymorphic microsatellites, i.e., non-coding DNA sequences consisting of tandem repeats and exhibiting high mutation rates (Jehle and Arntzen, 2002). By combining several microsatellite markers it is possible to estimate genetic differentiation among adjacent populations (Beebee and Rowe, 2008). Linear barriers, such as roads or major rivers, can cause a significant increase of genetic differentiation among amphibian breeding populations (Arens et al., 2007; Marsh et al., 2007). If agricultural fields function similarly as migration barriers or sink habitats, a population differentiation within a meta-population could be expected.

In the present study, we analyzed the genetic differentiation of six *Rana temporaria* LINNAEUS 1758 (European common frog) breeding pond populations from a viniculture landscape, using ten polymorphic microsatellite loci. Also, we analyzed four populations from the adjacent Palatinate Forest as a reference for widely unhindered gene flow. We tested for significant correlation of genetic population differentiation and landscape elements, including land use and linear barriers (roads and their associated traffic intensity), to explain the genetic structure in the study area. If viniculture acts as a migration limiting barrier for amphibians, we would reject the null hypothesis of a meta-population in the study area and rather expect a detectable genetic structuring among the analyzed *R. temporaria* breeding pond populations. Also, we compared the estimated genetic differentiation with the results of a landscape permeability model from the same study area (Lenhardt et al., 2013). In this model, pesticides were considered to decrease the permeability of agricultural land, causing a fragmentation or even isolation of amphibian breeding sites. The aim of the present study was to test the model predictions for the common frog by applying landscape genetic methods, i.e., whether the genetic differentiation

of the examined breeding pond populations would reflect the predicted population fragmentation of common frogs in the vinicultural landscape.

7.4.3 Material and Methods

The study was conducted in Rhineland-Palatinate, Germany, between Neustadt/Weinstrasse and Landau/Pfalz (Figure 10; see Appendix Figure 12, Figure 13 and Figure 14). We sampled ten breeding pond populations of *R. temporaria* during the breeding seasons 2012–2014. Six of these ponds (P1–P6) were located in the vineyards of Southern Palatinate and four (P7–P10) were inside the adjacent Palatinate Forest.

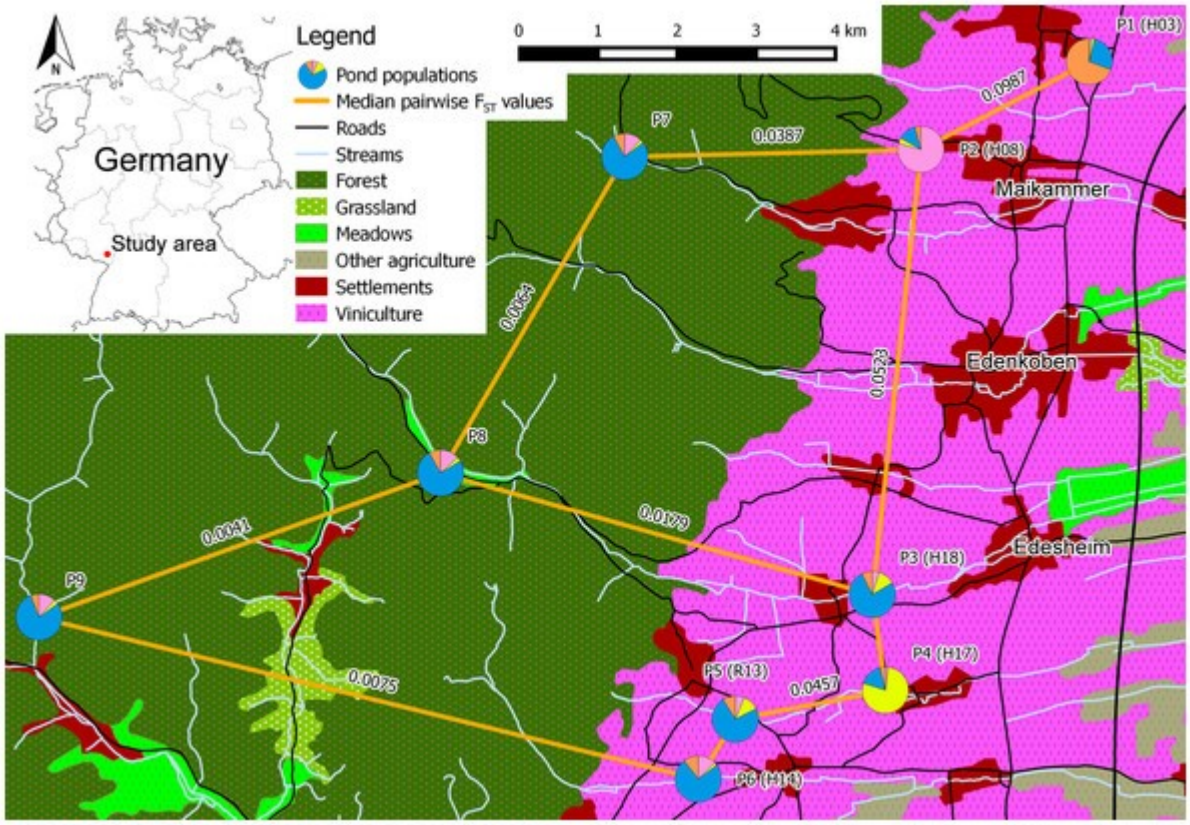

Figure 10 Schematic overview of the core study area in southern palatinate between “Neustadt an der Weinstraße” (north of P1) and “Landau in der Pfalz” (south of P6) with median pairwise F_{ST} values for selected pond population pairs. Pond labels of Lenhardt et al. (2013) in brackets. Pie charts of the pond populations show the overall share of each cluster on the population, based on the STRUCTURE analysis for clusters K = 4 (see Fig. 2 for cluster colors in pie charts).

The distance between the sampled ponds P1–P9 varied between about 0.9 and 15 km, whereas P10 was located about 40 km northwest of the core study area near Kaiserslautern (see Appendix Figure 15). The waterbodies of breeding pond

populations P3, P5 and P6 were directly connected to the Palatinate Forest by permanent or seasonal streams, whereas for P1, P2 and P4 this was not the case.

We collected eggs from all explicit distinguishable clutches (N = 7–10) of *R. temporaria* per breeding pond (P1–P9; in total 71 clutches) and hatched them in 300 ml glass bottles filled with tap water to Gossner stages 20–25. Sampling was approved by the Structure and Approval Directorate South of Rhineland-Palatinate, department 42, Upper nature conservation authority (approval number 42/553-254). Three tadpoles per clutch were randomly selected for genetic analysis. Since females of *R. temporaria* typically lay a single clutch per breeding season (Schlüpmann et al., 1996), we assumed only full-siblings existed within clutches. Furthermore, we included genetic data of 21 adult *R. temporaria* from P10 from a previous study (Müller et al., 2013). We applied a high salt DNA extraction protocol to obtain DNA from tissue samples of the tadpoles (Aljanabi and Martinez, 1997).

Table 8 Basic information on used microsatellites: amplification success (AS) based on all data as well as the number of sampled alleles and allelic richness for forest (F) and viniculture (V) populations. Physically unlinked loci are marked italic (see Cano et al., 2011).

Locus	***BFG130***	***BFG092***	**BFG066**	**BFG151**	***BFG090***	**BFG082**	***BFG099***	***BFG160***	***BFG145***	**BFG129**
Motif	TCTT	TATC	AAG	GAAA	CTAT	TATC	ACTC	TCTA	TCTA	CTAT
AS [%]	100	84	87	93	78	96	99	100	96	96
Number of alleles sampled										
F	7	22	17	20	16	21	5	23	16	25
V	7	19	13	23	13	22	4	23	15	23
Allelic richness										
F	6.924	21.759	16.195	19.762	16.000	20.665	4.928	22.578	15.928	24.638
V	6.914	17.635	12.952	20.738	13.000	19.900	4.000	19.992	14.115	21.513

We analyzed ten variable microsatellite loci (Table 8; Matsuba and Merilä, 2009) and amplified the fragments in two multiplex PCRs using the QIAGEN Multiplex PCR Kit (Hilden, Germany) following Müller *et al.* (2013). The selected loci were chosen from a number of tested loci due to their amplification success and polymorphism in an earlier study (Müller et al., 2013). Also, six of the selected loci were located on different chromosomes (Table 8; see Cano et al., 2011). Amplification products were run on a CEQ 8000 Sequencer (Beckman Coulter, Krefeld, Germany). Fragments were analyzed with the software GeneMarker 1.95 (SoftGenetics, State College,

Pennsylvania, USA) and verified with Micro-Checker 2.2.3 (Van Oosterhout et al., 2004).

The main concern of larvae sampling is a potential bias of the results due to siblings in the data set. Removing full-siblings most likely produces results that are closer to those calculated from adult individuals and therefore improves the inference of population genetic studies based on larval samples (Goldberg and Waits, 2010). We removed full-siblings from the data by randomly selecting one tadpole per clutch, resulting in seven to ten individuals per population. We calculated Hardy-Weinberg-Equilibrium over all populations using GenePop 4.2 (Raymond and Rousset, 2004). We grouped individuals from populations P1–P6 into a viniculture population (V) and individuals from P7 to P10 into a forest population (F) and calculated the number of sampled alleles (N_A) and allelic richness (N_{AR}) using FSTAT 2.9.3.2 (Goudet, 2001).

The removal of full-siblings from data may improve the quality of the results, but causes a low number of individuals per site, especially in small populations. This might introduce a bias due to picking one individual over another. To compensate for this potential bias, we applied the repeated randomized selection of genotypes (RRSG) approach (Lenhardt and Theissinger, 2017). This approach for removing full-siblings from an offspring data set produces population estimates which are closer to estimates calculated for the parental data set, compared to estimates based on data containing siblings. Any potential bias due to selection of one sibling over another is compensated by performing multiple estimates of the genetic parameters. This RRSG approach was thus applied in all subsequent population genetic analyses.

To examine the genetic structure of the sampled populations, the Bayesian clustering software STRUCTURE 2.3.4 (Pritchard et al., 2000) was used. Since the presence of siblings can also bias the detection of genetic clusters (Anderson and Dunham, 2008; Rodriguez-Ramilo and Wang, 2012), we again applied a RRSG approach creating 500 subsets of genotypes without siblings, resulting in 71 individuals from the populations P1–P9 per subset. Population P10 was excluded due to a possible isolation by distance effect (see results; Pritchard et al., 2010).

As we expected some genetic exchange between populations, but an overall weak population structuring, we chose the admixture model with imposed sampling locations (LOCPRIOR). The model was calculated with an initial burn-in of 100, 000 and a Markov Chain Monte Carlo (MCMC) of 500, 000 repeats for each subset and each predefined cluster number K between 1 and 9. To determine the most likely

number of clusters K, the program STRUCTURE HARVESTER (Earl and VonHoldt, 2012) was used. Results were combined with the LargeKGreedy algorithm with 10, 000 random input orders in CLUMPP (Jakobsson and Rosenberg, 2007) and visualised with DISTRUCT (Rosenberg, 2004).

For linkage disequilibrium over all populations, population pairwise F_{ST} and R_{ST} as well as for observed (H_O) and expected (H_E) heterozygosity calculations we applied the RRSG approach with 100,000 calculations using GenePop. Only one individual genotype per clutch was automatically selected in each calculation, thus producing results for linkage disequilibrium, F_{ST}, R_{ST}, H_O and H_E values based on data without full-siblings. For interpretation, we used median pairwise F_{ST} (MPF) and median pairwise R_{ST} (MPR) values as well as median H_O and H_E values over all RRSG calculations. For the interpretation of the linkage disequilibrium we, calculated a possibility of linkage for each loci pair by forming a quotient of number of calculations where linkage was detected (p-value ≤0.05) divided by total number of calculations of the RRSG approach. We considered a loci pair linked when 5% or more of the 100,000 calculations detected a statically significant linkage disequilibrium for the respective loci pair.

We calculated a distance matrix for the breeding ponds and analyzed isolation by distance for MPFs and MPRs over all breeding pond pairs using Genepop's subprogram ISOLDE (Rousset, 2008). We used MPF/(1-MPF) and MPR/(1-MPR) as the dependent variable and the corresponding linear geographic distance, number of roads as well as the cumulated traffic intensity of all roads (vehicles per 24 h; received from the Ministry of the Interior, Sports and Infrastructure Rhineland-Palatinate in 2015; see Appendix Table 13 and Table 14) between breeding ponds as the independent variable in a Mantel's test with Spearman rank correlation for matrix correlation with 10, 000 permutations (Rousset, 1997).

To address the spatial configuration of habitat types between breeding ponds, we adjusted the linear geographic distance with respect to present habitat types. Therefore, we obtained land cover data (ATKIS) of the study area from the State Office for Surveying and Geobasisinformation Rhineland-Palatinate (2015). We calculated the area of habitat types (settlements, viniculture, grassland, meadows, copse, forest and waterbodies) and length of roads in a 200 m wide strip between breeding ponds. Since the vinicultural study area has, apart from of the ponds and their surrounding areas, no mentionable hideout and hibernation options for

amphibians, we limited our analysis of the spatial configuration to the most direct migration routes for amphibians between ponds. Assuming an average daily migration distance of 100 m (Berger et al., 2011), 200 m wide strips take possible deviations from this average daily migration distance, resulting for example from foraging, into account (see also Arens et al., 2007; Vos et al., 2001).

Positive habitat types like grassland, meadows, copse, forest and waterbodies may increase the daily migration distance of amphibians due to favorable migration conditions (such as food availability, humidity and protection against predators). On the other hand, negative habitat types like settlements and viniculture may decrease the daily migration distance due to unfavorable migration conditions. In a weighted distance model, such positive and negative effects of habitat types on the migration of amphibians between breeding ponds can be addressed. We adapted a weighted distance model (Arens et al., 2007; Vos et al., 2001), which corrects the linear geographic distance based on the negative and positive habitat types between breeding ponds. We introduced a habitat correction factor into the model (Table 9), since each habitat type may impact the genetic differentiation with a different magnitude.

Table 9 Overview of all weighted distance models.

Weighted distance models	Description
$LGD*R_{NA}$	Linear geographic distance (LGD) weighted for the fraction of negative area (NA). R_{NA} being the negative area relative to the total area (TA) in a strip of 200 m wide between two ponds. Adjusted with the habitat correction factor (HCF) $R_{NA} = (NA * HCF + TA)/TA$
$LGD*R_{PA}$	Linear geographic distance (LGD) weighted for the fraction of positive area (PA). R_{PA} being the positive area relative to the total area (TA) in a strip of 200 m wide between two ponds. Adjusted with the habitat correction factor (HCF) $R_{PA} = TA/(PA * HCF + TA)$
$LGD*R_{NA}*R_{PA}$	Combined weighted distance for positive and negative area.

For each habitat type, we calculated the corrected linear geographic distance using the weighted distance model with a habitat correction factor from 1 to 100 in steps of 0.1. We selected the relevant habitat correction factor based on the highest R^2 of MPF as well as MPR and the corrected linear geographic distance. Afterwards, we used ISOLDE to analyze isolation by distance for MPFs as well as MPRs and the corrected linear geographic distance with the relevant habitat correction factor

provided by the weighted distance model, for each habitat type separately. Finally, we combined all habitats (see Table 9 and Table 10) that showed statistically significant isolation by distance in the individual weighted distance models into one weighted distance model and analyzed isolation by distance for MPFs as well as MPRs using ISOLDE.

Table 10 Results of isolation by distance for median pairwise F_{ST} (MPF) as well as median pairwise R_{ST} (MPR) and the linear geographic distance (LGD) corrected by the weighted distance models with habitat correction factor (HCF).

	MPFs			MPRs		
Weighted distance model	**HCF**	**p-value**	**R^2**	**HCF**	**p-value**	**R^2**
LGD*R_{NA} viniculture	10.8	<0.001	0.327	7.3	0.008	0.159
LGD*R_{NA} settlements	88.5	0.125	0.107	1.0	0.153	0.040
LGD*R_{PA} forest	8.8	0.005	0.303	4.0	0.016	0.079
LGD*R_{PA} grassland	16.2	0.365	0.043	38.5	0.239	0.069
LGD*R_{PA} meadows	11.6	0.165	0.302	10.3	0.092	0.140
LGD*R_{PA} copse	1.0	0.288	0.031	1.0	0.143	0.040
LGD*R_{PA} waterbodies	97.0	0.316	0.038	1.0	0.137	0.041

7.4.4 Results

We detected deviation from Hardy-Weinberg-Equilibrium due to heterozygote deficits on two loci (BFG082 and BFG129) over all populations. Forest populations showed higher values for number of sampled alleles and allelic richness in comparison to population viniculture (Table 8). Over all populations, we detected linkage disequilibrium for 27 out of 45 loci pairs (see Appendix Table 15). The highest percentage of linkage disequilibrium was detected for the locus pair BFG66 & BFG90 (95%). Also, we detected linkage disequilibrium for loci pairs that are physically unlinked (i.e., located on different chromosomes, Cano et al., 2011), for example BFG90 & BFG145 (86%), BFG90 & BFG160 (78%) and BFG92 & BFG145 (68%).

STRUCTURE HARVESTER identified $K = 4$ as the most meaningful number of clusters in our data set (see Table 11 and Appendix Figure 16). For $K = 4$, we detected for the breeding pond populations P1, P2 and P4 separate clusters, whereas the remaining populations formed a joined cluster. With an increased K ($K = 5$ to $K = 9$), P1, P2 and P4 still formed individual clusters, while the rest of the populations where assigned to the same cluster up to $K = 7$ (Figure 11).

Table 11 Expected and observed heterozygosity calculated with the repeated randomized selection of genotypes (RRSG) approach over all loci for breeding pond populations P1 to P10.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
H_E	0.852	0.685	0.776	0.722	0.703	0.664	0.788	0.831	0.840	0.824
H_O	0.757	0.600	0.643	0.560	0.629	0.514	0.657	0.771	0.778	0.738

Table 12 Results of the repeated randomized selection of genotypes (RRSG) approach for the median pairwise F_{ST} (MPF) and median pairwise R_{ST} (MPR). Populations 1 to 6 were located within vineyards, populations 7 to 10 in the Palatinate Forest. Population 10 was about 40 km away from the core study area.

		MPR									
	Pop.	1	2	3	4	5	6	7	8	9	10
MPF	1	--	0.1137	0.0104	0.0471	0.0022	0.0333	0.0518	0.0449	0.0826	0.1403
	2	0.0987	--	0.0851	0.0854	0.0577	0.0866	0.0277	0.0221	0.0000	0.0607
	3	0.0559	0.0523	--	0.0006	0.0000	0.0016	0.0005	0.0405	0.0333	0.0176
	4	0.0802	0.0781	0.0372	--	0.0000	0.1027	0.0975	0.0471	0.0355	0.0872
	5	0.0532	0.0519	0.0215	0.0457	--	0.0108	0.0536	0.0018	0.0260	0.0640
	6	0.0672	0.0383	0.0224	0.0540	0.0268	--	0.0093	0.0537	0.0572	0.0607
	7	0.0575	0.0387	0.0223	0.0479	0.0266	0.0012	--	0.0000	0.0000	0.0000
	8	0.0574	0.0418	0.0179	0.0459	0.0191	0.0123	0.0064	--	0.0000	0.0451
	9	0.0441	0.0339	0.0135	0.0410	0.0084	0.0075	0.0159	0.0041	--	0.0103
	10	0.0687	0.0708	0.0328	0.0751	0.0434	0.0374	0.0409	0.0212	0.0265	--

With exception of P1 (H_E = 0.852), all pond populations located in viniculture showed lower levels of heterozygosity over all loci (H_E = 0.663–0.776) than populations located in the Palatinate Forest (P7–P10; H_E = 0.788–0.840; Table 11). MPFs ranged from 0.0012 to 0.0987 and MPRs from 0.0000 to 0.1403 (Table 12).

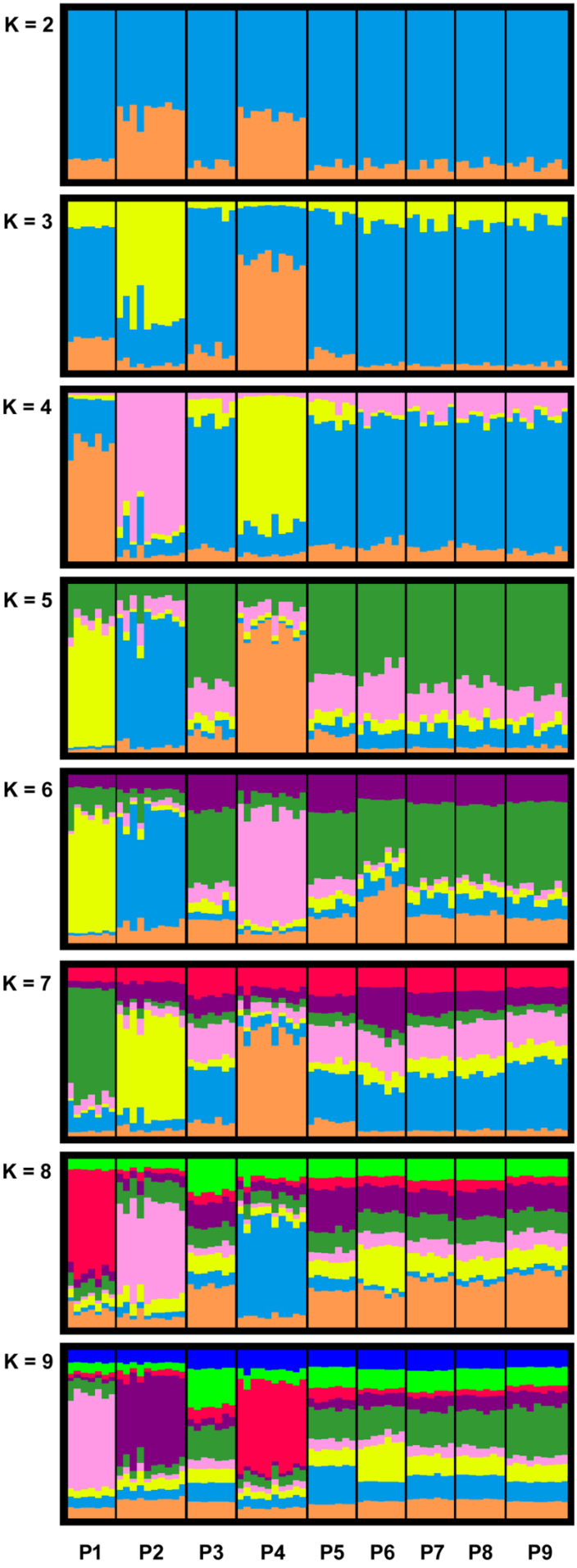

Figure 11 Bar plots of combined STRUCTURE analysis for clusters K = 2 to K = 9 of the investigated *R. temporaria* breeding pond populations in the study area. STRUCTURE HARVESTER identified K = 4 as the most meaningful number of clusters. Each vertical bar represents one individual, and the color composition visualizes the probability to belong to one of the K clusters defined by STRUCTURE. P10 was excluded from the analysis due to the different life stage of the samples.

The highest MPF and MPR were estimated between P1 and P2 at a linear geographic distance of 2.4 km (see Appendix Table 17 for a matrix of all linear geographic distances). The lowest MPF was found between P6 and P7 at a linear geographic distance of 7.9 km. On average, genetic differentiation between population pairs in viniculture (average MPF = 0.0523, average MPR = 0.0425) was higher than between population pairs in forest or forest and viniculture, whereas population pairs in the Palatinate Forest showed the lowest MPFs and MPRs (average MPF = 0.0192, average MPR = 0.0092). In general, genetic differentiation among breeding pond populations in viniculture was comparatively high, despite close proximity of the breeding ponds (e.g., linear geographic distance <1 km: MPF = 0.0467; linear geographic distance <2.5 km: MPF = 0.0987 and MPR = 0.1027), as opposed to breeding pond populations in the forest (linear geographic distances = 4.5–9.5 km; MPFs = 0.0064–0.0409 and MPRs = 0.0041–0.0648). Yet populations at breeding ponds with a direct connection to the Palatinate Forest by permanent or seasonal streams exhibited lower MPFs to forest pond populations (P3, P5 and P6) compared with agricultural pond populations not connected to the forest (P1, P2 and P4; see Table 12).

Over all breeding pond populations, ISOLDE detected no statistically significant relation between MPFs or MPRs and linear geographic distance, number of roads or accumulated traffic intensity between population pairs ($p > 0.050$). Isolation by distance was statistically significant for MPFs of the four forest populations ($p = 0.0320$). However, when excluding the most distant population P10, isolation by distance was no longer statistically significant.

When analyzing the linear geographic distances corrected by the weighted distance model, isolation by distance was statistically significant (p-values < 0.050) for viniculture and forest (MPF and MPR; Table 10). Corrected linear geographic distances of viniculture, forest and meadows showed an explained variance of more than 0.300 when correlated with MPF, whereas explained variance was significantly lower when correlated with MPR (0.079–0.159). Combining all distance corrections (R_{NA} viniculture and R_{PA} forest, see Table 9 and Table 10) that showed statistically significant isolation by distance into one weighted distance model resulted in statistically significant isolation by distance for MPF as well as MPR (p-values < 0.005).

7.4.5 Discussion

We analyzed the genetic differentiation of *R. temporaria* of breeding pond populations within viniculture and the Palatinate Forest to investigate potential genetic population differentiation due to agricultural land use. Our microsatellite data exhibited linkage disequilibrium for 27 of the 45 loci pairs. However, high percentages (up to 95%) of linkage disequilibrium were also detected for multiple loci pairs located on different chromosomes and for which linkage is thus unlikely. Moreover, the linkage calculations were performed over the whole dataset as one meta-population. This could have additionally affected the linkage analyses due to the underlying population structuring, since specific allele combinations might only occur in some fragmented populations, thus inferring linked inheritage of respective loci. The vice versa assumption, that genetically linked loci might have inferred the detected population fragmentation by structure as unreal signal in our data, can be rejected, since our analyses for gene flow among all populations (MPFs and MPRs, Table 12) also suggested that the fragmented populations P1, P2 and P4 were more isolated compared to the other populations. Thus, we evaluated the detected linkage disequilibrium as statistical artefact and decided to use all ten loci for subsequent analyses.

Our analysis showed structuring within the investigated breeding pond populations and highlighted breeding pond populations P1, P2 and P4 (all located in viniculture) as isolated from the meta-population (Figure 11). Moreover, our data exhibited higher genetic differentiation among breeding pond populations in the agricultural landscape compared with breeding pond populations in the Palatinate Forest (Table 12). We observed the highest genetic differentiation between breeding pond populations in viniculture, which were only a few kilometers apart (e.g., P1 and P2 with a linear geographic distance of less than 2.5 km: MPF = 0.0987 and MPR = 0.1137). The most distant forest population P10 was responsible for a significant isolation by distance among the forest populations. However the results for P10 have to be treated with caution, since we mixed different life stages and generations, which may introduce some bias (Peterman et al., 2016). Still, even when we exclude P10 from the data set, the genetic differentiation within the remaining forest populations was lower compared with viniculture populations.

Breeding pond populations in the agricultural landscape with a direct connection to the Palatinate Forest by permanent or seasonal streams exhibited

lower MPFs to forest pond populations compared with agricultural pond populations not connected to the forest (Table 12), indicating the importance of waterbodies including the adjacent riparian vegetation for the genetic connectivity in amphibian breeding pond populations. In 2012, we observed the translocation of *Rana temporaria* clutches at P8, which were intentionally moved into the nearby stream due to drought by staff of the “Modenbacher Hof”, a close-by horse ranch. This stream is connected directly to P3. During major rain events, some of the clutches could have been flushed into the pond at P3. Surviving amphibians could then have contributed to the following reproduction phases, resulting in a one directional genetic exchange and explaining the rather low MPF value of 0.0179 between P3 and P8.

Our population genetic results were similar to the differentiation of *Rana arvalis* (F_{ST} = 0.06) in Noord-Brabant, Netherlands, where landscape permeability was low due to farming intensity and urbanization (Arens et al., 2007; Van der Sluis and Vos, 1997). Additionally, breeding sites in Noord-Brabant became polluted with agrochemicals (pesticides and fertilizers) as a result of intensive agriculture (Hoogerwerf and Crombaghs, 1993). For *R. temporaria*, Safner et al., (2011) found F_{ST} values between 0.024 and 0.193 in a human dominated landscape near Chambery, France, on a fine spatial scale (<20 km). Negative effects of high agricultural intensity on the occurrence, abundance and genetic diversity of amphibians on a regional and national scale were also found in several other studies (Johansson et al., 2005; Trochet et al., 2016; Youngquist et al., 2016).

Our analyses in ISOLDE rejected isolation by distance based on roads and associated traffic intensity as the sole explanation of the genetic differentiation of *R. temporaria*; although, an effect of roads on amphibian population connectivity has been shown in other studies (Beebee, 2013; Buskirk, 2012; Krug and Pröhl, 2013). However, the weighted distance model showed significant isolation by distance for viniculture and forest, indicating that these two habitat types are the most relevant parameters to explain the structuring of breeding pond populations in the study area. Also, the introduction of the habitat correction factor to the weighted distance model showed that applying habitat specific permeability can improve the detection of isolation by distance remarkably. However, the habitat correction factor has to be interpreted in context with the explained variance in the isolation by distance analyses, since a high habitat correction factor not necessarily translates into a high impact on population differentiation when explained variance is low (<0.1). With

exception of habitat type copse, introducing the habitat correction factor to the weighted distance model did improve the explained variance of the corrected linear geographic distance, when correlated with MPF. For MPR, settlements, copse and waterbodies did not benefit from the introduction of the habitat correction factor.

Lenhardt et al., (2013) assessed the potential fragmentation of breeding sites in the same study area with a simplified expert based landscape permeability model. They predicted fragmentation, and therefore a potential genetic differentiation, of agricultural breeding ponds in close proximity, when pesticide applications were considered as a migration limiting model factor. Our genetic data presented here confirmed the predicted fragmentation of P1 from the other breeding pond populations (MPFs from 0.0553 to 0.0987; Table 12). However, the model in Lenhardt et al., (2013) overestimated the potential fragmentation of breeding sites in a number of cases, especially when the breeding ponds in viniculture (e.g., P3 and P6, Figure 10) were directly connected to the Palatinate Forest via permanent streams. Thus, permanent streams and their riparian vegetation may serve as suitable migration or dispersal corridors within the agricultural landscape.

In our study area, the intensification of viniculture started in the early 20th century. Particularly in the last 50 to 80 years, the development of mechanical equipment and the broad availability of pesticides have led to a further intensification and expansion of viniculture, leaving amphibian species like *Rana temporaria* with small fragmented breeding habitats within the agricultural landscape. Nowadays, typical application scenarios in vineyards of Southern Palatinate consist of up to 12 (on average 8) fungicide applications per year, within intervals of about 10–14 days between early May and mid-August (Lenhardt et al., 2013; Roßberg, 2009). During this period, amphibians are in their terrestrial life stage and juvenile individuals migrate away from the spawning waters. Furthermore, fungicide applications are often applied before or after rain events of more than 3 mm precipitation (Lenhardt et al., 2013). Such rain events may trigger amphibian migration and general amphibian activity (Baldwin et al., 2006; Rothermel, 2004). Therefore, the spatial and temporal overlap of amphibians and applied fungicides is very likely.

Since *R. temporaria* becomes sexually mature in the third (rarely second or first) year of life (Westheide and Rieger, 2015), about 25–40 overlapping generations have passed since the intensification of viniculture started. Due to the few passed generations, overall population differentiation is still moderate (F_{ST} between 0.05 –

0.15; Hartl and Clark, 2007; Wright, 1978) but may increase due to time-delay in genetic differentiation (Bossart and Pashley Prowell, 1998). Also, F_{ST} might already underestimate the current genetic differentiation when polymorphic loci are used in highly structured populations, since F_{ST} can't distinguish between mutation and dispersal (Balloux and Lugon-Moulin, 2002). The genetic differentiations identified by MPF values were supported by the estimated MPR values (Table 12), which underlines a separation of breeding pond populations in the study area.

Due to the temporal coincidence of amphibian activity and pesticide applications, negative effects on meta-population dynamics could be expected in a viniculture landscape, if fungicides are generally of high toxicity and exposure of amphibians is high. Also, pesticide applications were the most frequent management measures in viniculture (up to 12 applications) and can affect amphibians not only on the application day, like tillage operations, but up to several days after application, depending on the chemical decomposition of pesticides. Recent studies and surveys confirmed the presence of pesticides in amphibian habitats and waterbodies in general (Smalling et al., 2012; Ulrich et al., 2015), as well as in amphibian tissues (Battaglin et al., 2016; Cusaac et al., 2016; Smalling et al., 2015, 2013). Furthermore, pesticide concentrations in amphibian tissues were positively correlated with agricultural and urban land around breeding sites (Battaglin et al., 2016). Therefore, pesticides may be a major factor for the detected genetic differentiation within the investigated *R. temporaria* breeding pond populations. Yet we can only assume this impact and want to highlight the need of more detailed studies on the effects of pesticides on natural amphibian populations, taking different life stages as well as different species into account.

We were not able to address differences between organic and conventional viniculture, since reference breeding sites with noteworthy portions of organic viniculture were not available in or nearby the study area. Also, it is currently unclear if the use of copper and sulfur within organic viniculture would actually improve the overall situation for amphibians (Mackie et al., 2013; Milanovi et al., 2013).

In contrast to our and others findings, some studies observed no impact of agricultural land use on the genetic differentiation of amphibians, although the investigated amphibian species were known to forage in intensively managed agricultural areas (Frei et al., 2016; Le Lay et al., 2015). Also, some level of pesticide tolerance for amphibians from agricultural breeding pond populations was detected

(Hua et al., 2015, 2013). Yet such findings should not be generalized, since tested taxa and pesticides were limited, and pesticides still may cause lethal or sublethal effects on amphibians, depending on the path of exposure, exposure level and amphibian life stage.

Although *R. temporaria* is considered 'not endangered' in Germany (Kühnel et al., 2009) and 'least concerned' in Europe (Temple and Cox, 2009), amphibian census indicated that many breeding pond populations, especially in agricultural land, were rather small (one to ten clutches) and populations with more than 150 clutches were generally rare (Schlüpmann et al., 2004, 1996; Wolfbeck et al., 2007). Consistent with these observations, amphibian surveys in the study area counted between 1 and 60 clutches per breeding site during 2007–2010 (S Bischoff, pers. comm., 2011; see Appendix Table 18). We repeatedly counted ten or less clutches for all breeding pond populations within viniculture (P1–P6) during our samplings from 2012 to 2014. Considering the small size of breeding pond populations in viniculture, local extinction may occur when breeding sites have a loose connectivity to surrounding terrestrial habitats (Safner et al., 2011).

Based on our results, we are concerned about the persistence of amphibians in agricultural areas, since we can recognize negative trends on the genetic diversity and differentiation of breeding pond populations. Typical visible barriers like roads with associated amphibian road mortality could not explain the genetic structuring of the breeding sites. Yet we could identify viniculture as a barrier for genetic exchange. Since pesticide applications are the most frequent management measure in viniculture and pesticides can cause high mortalities in amphibians, pesticides may have a major impact on amphibian dispersal and therefore on genetic exchange between breeding sites. Following the precautionary principle it may be advisable to reduce or avoid pesticide applications during amphibian migration phases and to mitigate pesticide contamination of amphibian breeding ponds. We recommend further research on the impact of pesticides on amphibian individuals and populations in agricultural landscapes.

7.4.6 Appendix A

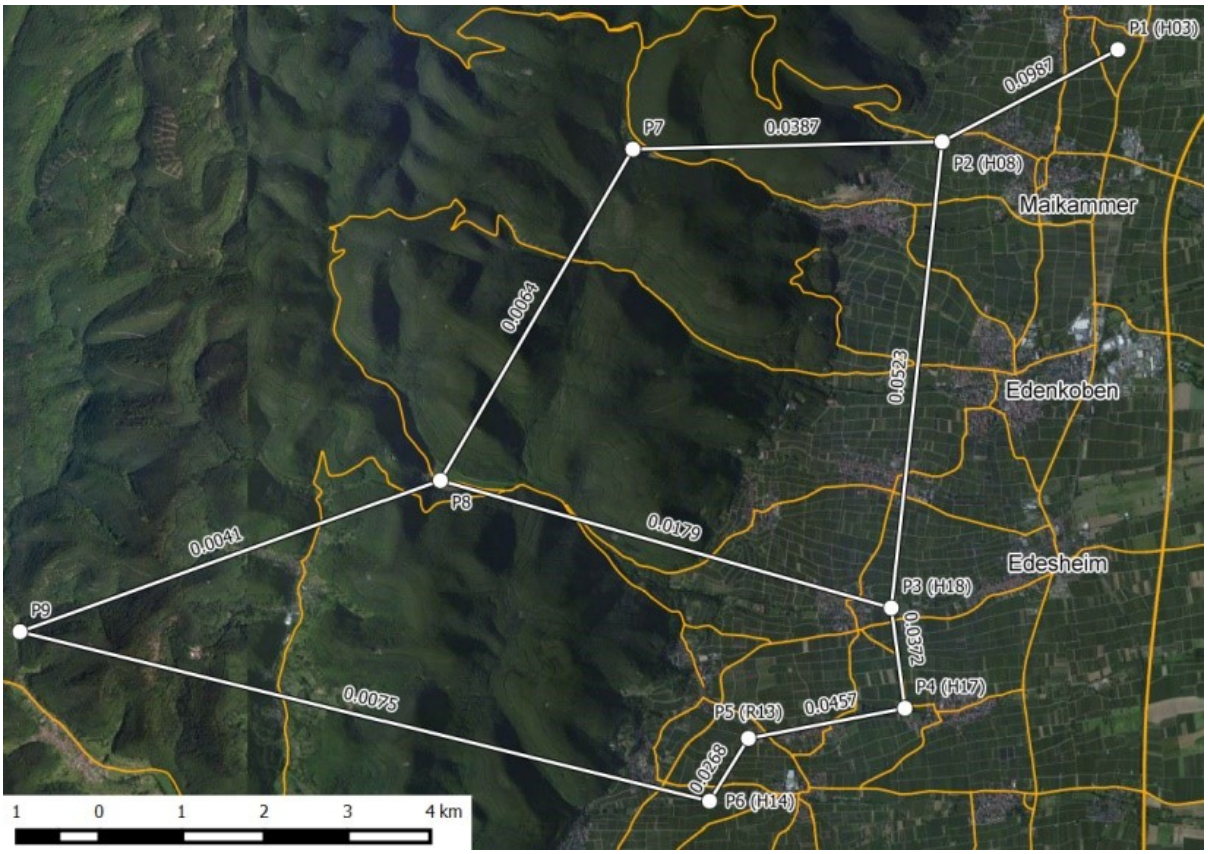

Figure 12 Aerial photo of the core study area between "Neustadt (an der Weinstraße)" (north of P1) and "Landau (in der Pfalz)" (south of P6) with selected median pairwise F_{ST} values. Relevant traffic infrastructure is highlighted in orange (aerial photo from Bing Maps, http://www.bing.com/mapspreview).

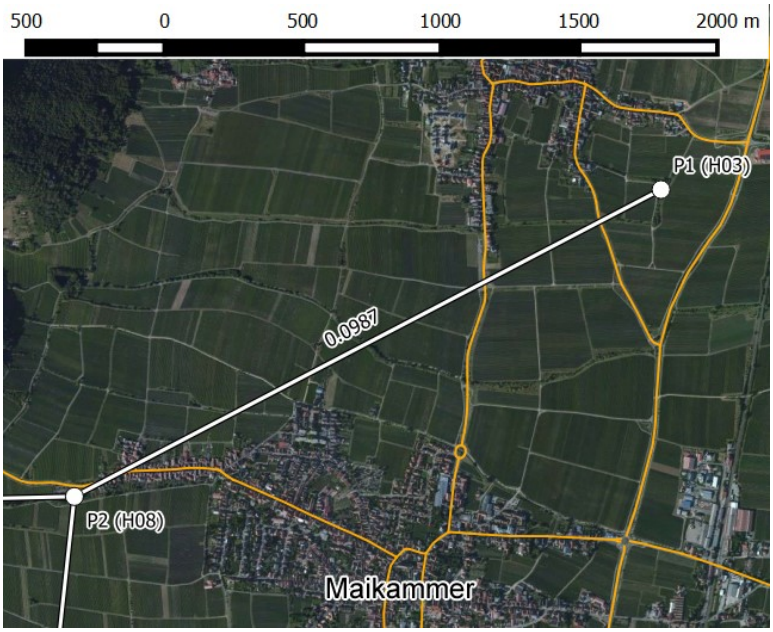

Figure 13 Close up aerial photo of landscape between breeding ponds P1 and P2 (2 394 m apart) in the north of the core study area with median pairwise F_{ST} value. Relevant traffic infrastructure is highlighted in orange (aerial photo from Bing Maps, http://www.bing.com/mapspreview).

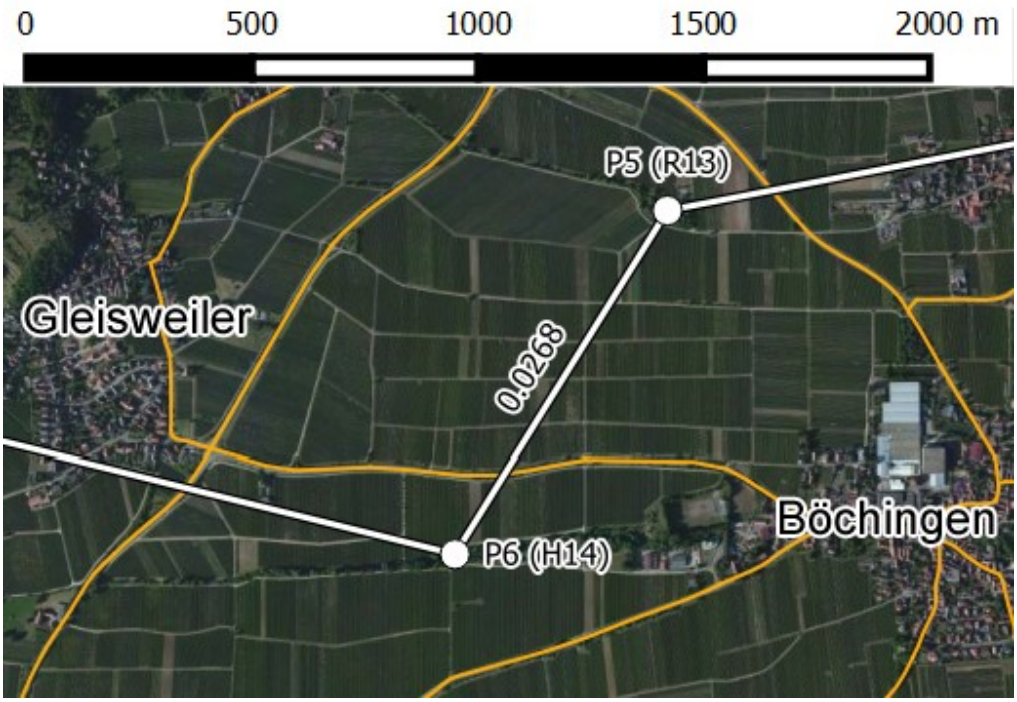

Figure 14 Close up aerial photo of landscape between breeding ponds P5 and P6 (890 m apart) with median pairwise F_{ST} value. Relevant traffic infrastructure is highlighted in orange (aerial photo from Bing Maps, http://www.bing.com/mapspreview).

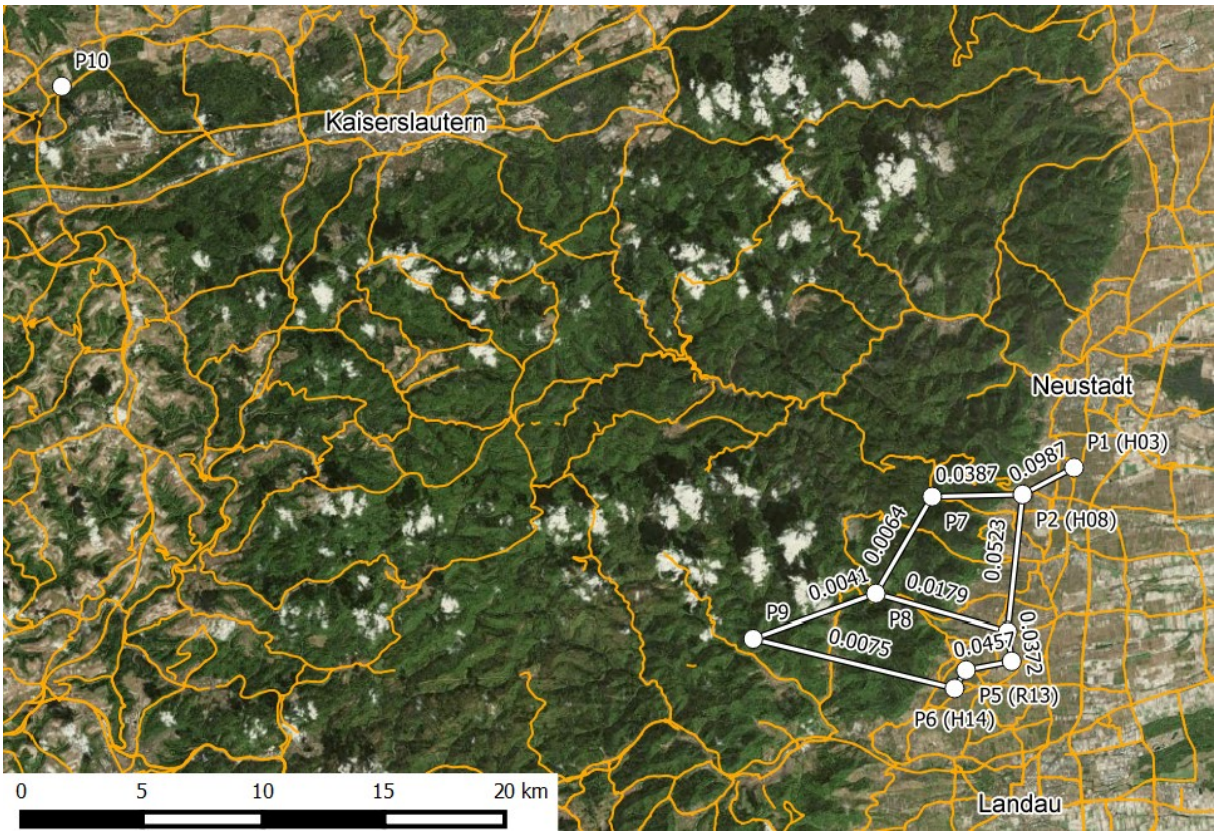

Figure 15 Aerial photo of the core study area between between “Neustadt (an der Weinstraße)” and “Landau (in der Pfalz)” with selected median pairwise F_{ST} values and P10 near Kaiserslautern. Relevant traffic infrastructure is highlighted in orange (aerial photo from Bing Maps, http://www.bing.com/mapspreview).

Table 13 Overview of all relevant roads in the study area. Traffic intensity (vehicles per 24 hours) were obtained from the Ministry of the Inner, Sports and Infrastructure in Rhineland-Palatinate (marked with an asterisk; Iris Honrath, personal communication) or estimated based on traffic intensity of nearby roads and geographical location.

Road	Type	Traffic (in 24h)	
A 6	motorway	62674	*
B 270	primary	14280	*
L 395	secondary	6000	
L 502	secondary	6000	
L 503	secondary	6259	*
L 369	secondary	6000	
L 356	secondary	6000	
K 32	tertiary	3148	*
K 5	secondary	3000	
K 53	tertiary	3000	
K 50	tertiary	3000	
B 48	primary	2934	*
L 506	secondary	2836	*
L 514	secondary	2632	*
L 499	secondary	2549	*
L 512	secondary	2500	
L 500	secondary	2000	
L 519	secondary	1955	*
L 513	secondary	1553	*
K 49	tertiary	1500	
K 55	tertiary	1500	

Road	Type	Traffic (in 24h)	
K 15	secondary	1500	
K 4	tertiary	1500	
L 505	secondary	1500	
L 507	secondary	1456	*
L 504	secondary	1217	*
K 57	tertiary	1067	*
K 40	secondary	1000	
K 38	tertiary	1000	
K 17	tertiary	1000	
K 18	tertiary	1000	
K 19	tertiary	1000	
K 51	unclassified	1000	
K 30	unclassified	931	
K 31	secondary	931	
K 6	tertiary	931	*
K 58	tertiary	729	*
K 59	tertiary	729	
L 515	secondary	645	*
K 78	unclassified	586	
K 56	tertiary	586	*

Table 14 Number of roads between population pairs and their accumulated traffic intensity (vehicles in 24 hours on all roads between the pairs).

		P1	P2	P3	P4	P5	P6	P7	P8	P9
Number of roads	P2	3								
	P3	7	6							
	P4	10	7	2						
	P5	9	8	4	2					
	P6	10	9	5	3	1				
	P7	4	2	3	5	5	7			
	P8	6	3	4	7	5	5	1		
	P9	6	4	3	4	3	4	1	2	
	P10	14	15	15	18	15	15	15	12	12
accumulated traffic intensity	P2	4645								
	P3	14192	12978							
	P4	17165	14089	2042						
	P5	16715	13730	5609	3567					
	P6	18268	15283	7162	5120	1553				
	P7	7277	3277	6267	8309	7019	10189			
	P8	9139	4208	6996	9767	8245	8731	931		
	P9	11044	7044	6065	7521	6247	7800	931	3767	
	P10	122690	123467	119481	124252	115788	116341	121691	109886	109955

Table 15 Linkage of loci pairs with RRSG approach. Percentage values indicate the relative number of runs out of 100,000 calculations where linkage was detected (p-value less or equal to 0.05).

Loci pair	Linkage detected out of 100,000 calculations [%]
BFG130 & BFG092	0.04
BFG090 & BFG129	0.72
BFG090 & BFG145	0.87
BFG090 & BFG082	0.34
BFG090 & BFG099	0.00
BFG090 & BFG160	0.08
BFG145 & BFG129	0.78
BFG082 & BFG129	0.40
BFG082 & BFG145	0.65
BFG082 & BFG099	0.00
BFG082 & BFG160	0.01
BFG092 & BFG090	0.74
BFG092 & BFG129	0.70
BFG092 & BFG145	0.69
BFG092 & BFG082	0.42
BFG092 & BFG066	0.24
BFG092 & BFG099	0.00
BFG092 & BFG151	0.74
BFG092 & BFG160	0.13
BFG130 & BFG090	0.00
BFG130 & BFG129	0.13
BFG130 & BFG145	0.19
BFG130 & BFG082	0.02

Loci pair	Linkage detected out of 100,000 calculations [%]
BFG130 & BFG066	0.00
BFG130 & BFG099	0.00
BFG130 & BFG151	0.00
BFG130 & BFG160	0.00
BFG066 & BFG090	0.96
BFG066 & BFG129	0.64
BFG066 & BFG145	0.18
BFG066 & BFG082	0.00
BFG066 & BFG099	0.02
BFG066 & BFG151	0.00
BFG066 & BFG160	0.04
BFG099 & BFG129	0.05
BFG099 & BFG145	0.04
BFG099 & BFG160	0.00
BFG151 & BFG090	0.59
BFG151 & BFG129	0.57
BFG151 & BFG145	0.81
BFG151 & BFG082	0.18
BFG151 & BFG099	0.22
BFG151 & BFG160	0.12
BFG160 & BFG129	0.59
BFG160 & BFG145	0.30

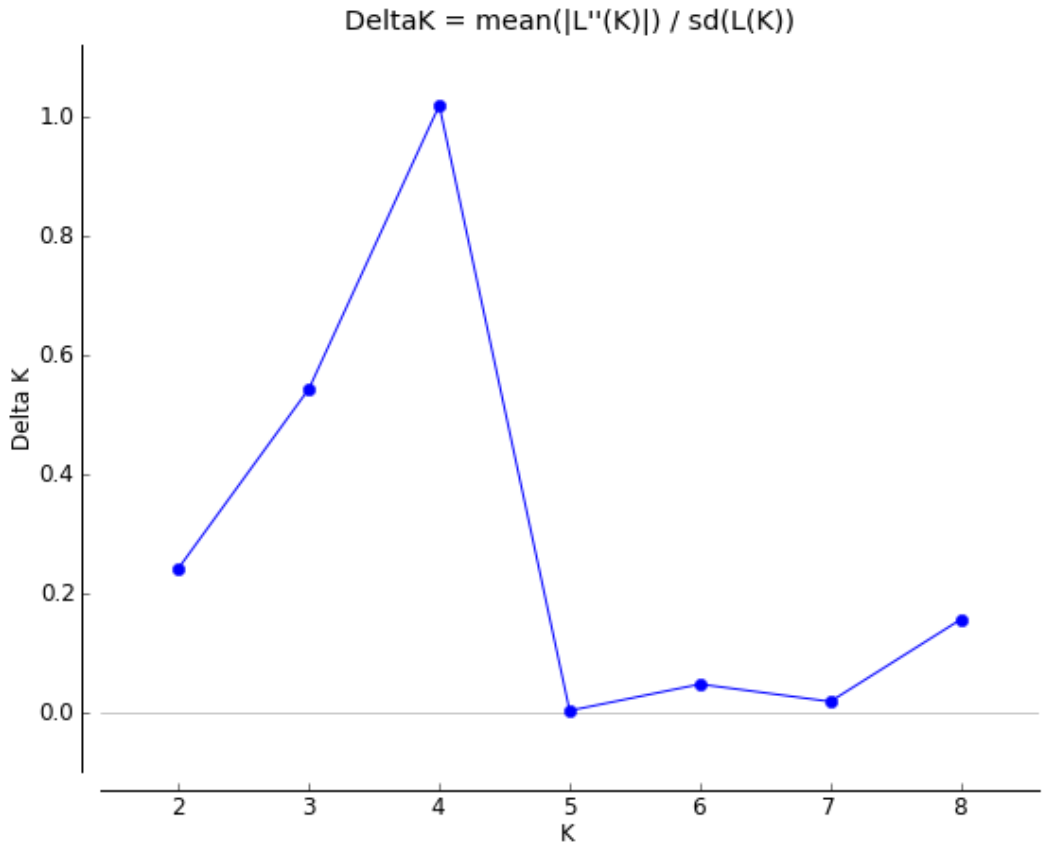

Figure 16 Plot of delta K values from the Structure analyses, obtained through STRUCTURE HARVESTER.

Table 16 Evanno table output from the Structure analyses, obtained through STRUCTURE HARVESTER.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	500	-3327.7386	43.1840	NA	NA	NA
2	500	-3291.5598	49.5511	36.178800	12.029200	0.242764
3	500	-3267.4102	56.2733	24.149600	30.581800	0.543451
4	500	-3273.8424	86.4383	-6.432200	88.074400	1.018928
5	500	-3368.3490	139.3570	-94.506600	0.514200	0.003690
6	500	-3463.3698	172.8867	-95.020800	8.314400	0.048092
7	500	-3566.7050	205.5675	-103.335200	3.897000	0.018957
8	500	-3666.1432	309.9213	-99.438200	48.534200	0.156602
9	500	-3717.0472	337.7702	-50.904000	NA	NA

Table 17 Linear geographical distance (LGD) in km between all population pairs.

		P1	P2	P3	P4	P5	P6	P7	P8	P9
LGD in km	P2	2.394								
	P3	7.254	5.644							
	P4	8.334	6.834	1.220						
	P5	9.415	7.553	2.335	1.926					
	P6	10.303	8.420	3.200	2.614	0.890				
	P7	5.979	3.737	6.342	7.489	7.227	7.901			
	P8	9.689	7.306	5.662	6.250	4.849	5.049	4.616		
	P9	14.992	12.598	10.526	10.728	8.890	8.573	9.403	5.387	
	P10	44.563	43.008	44.974	45.732	44.321	44.346	39.639	39.512	36.423

Table 18 Clutch counts at the monitored amphibian breeding sites with presence of Rana temporaria during breeding seasons 2011 to 2014 (na = not available).

Pond/Pop.	**2011**	**2012**	**2013**	**2014**
P1	8	7	7	0
P2	10	12	0	8
P3	9	11	8	0
P4	19	22	8	0
P5	7	8	0	0
P6	8	8	5	0
P7	26	32	27	24
P8	21	25	19	18
P9	> 100	> 100	> 100	> 100
P10	> 100	na	na	na

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9 Appendix

9.1 Declaration

I, the undersigned author of this work, declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

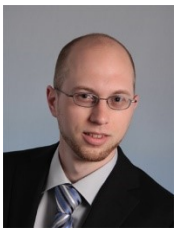
Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

15.12.2017, Patrick P. Lenhardt

9.2 Curriculum Vitae

Personal Information

Name:	Patrick P. Lenhardt
Date of birth:	04.07.1984 (Landau)
E-Mail:	lenhardt@uni-landau.de
Nationality:	German



Education and work experience

Since 2015	Software Developer at Dr. Knoell Consult GmbH
2012 - 2018	PhD student, Institute for Environmental Sciences, University of Koblenz-Landau. Working groups: Community Ecology & Ecotoxicology as well as Conservation Genetics Title of the dissertation: Amphibians in the agricultural landscape
2012 - 2015	Scientific and technical assistant at the University of Koblenz-Landau, Campus Landau
2011	Diploma in Environmental Sciences, University Koblenz-Landau, Campus Landau Title of the diploma thesis: Modellhafte Abschätzung der Ausbreitungsräume autochthoner Amphibien unter Berücksichtigung der Landschaftsstruktur und dem Einsatz von Pestiziden im Weinbau
2004 - 2011	Studies of Environmental Sciences • Major subject: Ecotoxicology, Biodiversity & Sustainability, Geoecology • Minor subject: Applied Ecology, Environmental Economics

Publications (Doctoral thesis)

Lenhardt P.P., Brühl C.A., Leeb C., Theissinger K., 2017. Amphibian population genetics in agricultural landscapes: does viniculture drive the population structuring of the European common frog (*Rana temporaria*)? *PeerJ* 5:e3520.

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Müller, A. S., **Lenhardt, P. P.**, & Theissinger, K. (2013). Pros and cons of external swabbing of amphibians for genetic analyses. *European Journal of Wildlife Research*, *59*, 609–612.

Further publications

Hahn M., **Lenhardt P. P.**, Brühl C. A. 2014. Characterization of field margins in intensified agro-ecosystems-why narrow margins should matter in terrestrial pesticide risk assessment and management. *Integrated Environmental Assessment and Management* 10. DOI: 10.1002/ieam.1535.

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Presentations at scientific conferences

Lenhardt P. P., Brühl C. A., Theissinger K. (2015): Are pesticides a factor for genetic differentiation of amphibian populations? Meeting of the SETAC Europe 2015, Barcelona, Spain

Lenhardt P. P., Brühl C. A., Theissinger K., Berger G. (2014): Amphibians and agriculture – chemical fragmentation of breeding pond populations. Meeting of the SETAC Europe 2014, Basel, Switzerland.

Lenhardt P. P. & Brühl C. A. (2010): Modellbasierte Abschätzung der potenziellen PSM-Exposition adulter Amphibien in der Agrarlandschaft (Weinbaugebiet Südpfalz). 4th joint Annual Meeting of the SETAC GLB and GDCh (Section Environmental chemistry and Ecotoxicology) 2010, Dessau, Germany

Hahn M., **Lenhardt P. P.**, Vollmar T., Brühl C. A. (2010): Erfassung der Breiten von Saumstrukturen landwirtschaftlicher Flächen auf digitalen Orthophotos (DOPs). Oral presentation at the 4th joint Annual Meeting of the SETAC GLB and the GDCh (Section Environmental chemistry and Ecotoxicology) 2010, Dessau, Germany.

Stahlschmidt P., **Lenhardt P. P.**, Swarowsky K. (2010). Profitieren Fledermäuse von künstlich angelegten Kleingewässern in der Agralandschaft? Poster Presentation, 4th joint Annual Meeting of the SETAC GLB and GDCh (Section Environmental chemistry and Ecotoxicology) 2010, Dessau, Germany

9.3 Own and co-author contributions

Table 19 Tabular overview of own and co-author contributions

Conception and Design	Field work	Laboratory work	Data evaluation	Interpretation	Wrote manuscript	Contributed to manuscript	Tutor
Müller, A. S., Lenhardt, P. P., & Theissinger, K. (2013). Pros and cons of external swabbing of amphibians for genetic analyses. European Journal of Wildlife Research, 59, 609–612.							
Theissinger, K **Lenhardt, P. P.**	Müller, A. S.	Müller, A. S. **Lenhardt, P. P.** Theissinger, K	Müller, A. S. **Lenhardt, P. P.** Theissinger, K	Müller, A. S. **Lenhardt, P. P.** Theissinger, K	Müller, A. S. **Lenhardt, P. P.**	Theissinger, K	Theissinger, K
Lenhardt, P.P., Brühl, C.A., Berger, G., 2015. Temporal coincidence of amphibian migration and pesticide applications on arable fields in spring. Basic and Applied Ecology 16, 54–63.							
Berger, G.	Berger, G.	-	Berger, G. **Lenhardt, P.P.**	Berger, G. **Lenhardt, P.P.** Brühl, C.A.	**Lenhardt, P.P.**	Berger, G. Brühl, C.A.	Brühl, C.A.
Lenhardt, P.P., Theissinger, K., 2017. Repeated randomized selection of genotypes for reliable estimates of population differentiation in data containing siblings. European Journal of Wildlife Research, 63, 8.							
Lenhardt, P.P.	-	**Lenhardt, P.P.**	**Lenhardt, P.P.**	**Lenhardt, P.P.** Theissinger, K	**Lenhardt, P.P.**	Theissinger, K	Theissinger, K
Lenhardt P.P., Brühl C.A., Leeb C., Theissinger K., 2017. Amphibian population genetics in agricultural landscapes: does viniculture drive the population structuring of the European common frog (*Rana temporaria*)? PeerJ 5:e3520.							
Lenhardt, P.P. Brühl C.A. Theissinger, K	**Lenhardt, P.P.**	**Lenhardt, P.P.**	**Lenhardt, P.P.** Leeb C. (STRUCTURE) Theissinger, K	**Lenhardt, P.P.** Leeb C. (STRUCTURE) Brühl C.A. Theissinger, K	**Lenhardt, P.P.**	Brühl C.A. Theissinger, K Leeb C.	Brühl C.A. Theissinger, K

9.4 Teaching involvement

During my PhD study at the University Koblenz-Landau, Campus Landau, I was involved in teaching, co-supervising and supervising students in the courses “Molecular Ecology II” (2012, 2013), “Methods of Environmental Science 2” (2013, 2014), “Methods of Environmental Science 3” (2014) and “Project Environmental Science” (2013, 2014). Furthermore, I conducted the workshops “An Introduction to Postgres & PostGIS” (2012) and “GIS Modellierung in der Ökologie und Ökotoxikologie” (2014).