Amphibians in a fragmented viticultural landscape

by

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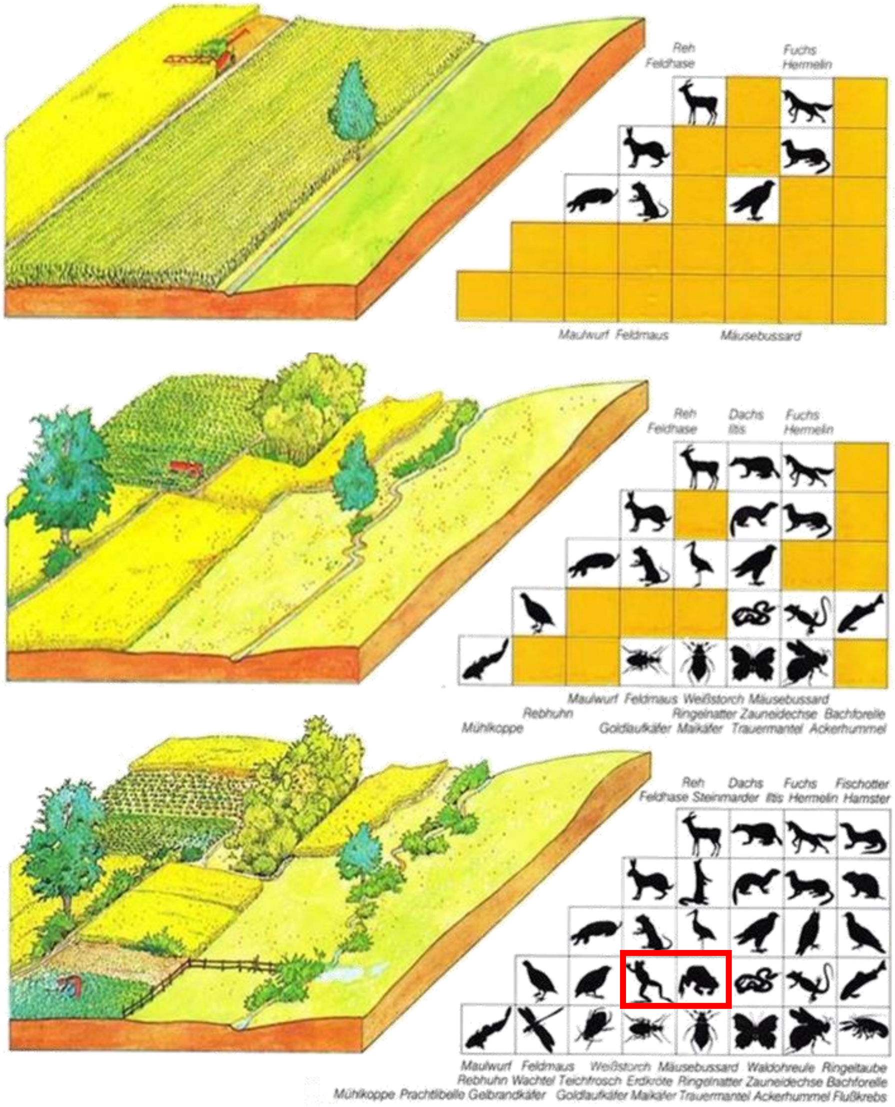

Bundesamt, F.U., & Landschaft, W. U. Umwelt in der Schweiz **1997**. Berna, Buwal. (modified)

It could be so easy.

Es geht immer ums Vollenden und den Superbowl.

Nino aus Wien

List of publications

The present thesis is a cumulative dissertation based on five peer-reviewed publications.

1. **Leeb, C.**, Brühl, C., Theissinger, K., 2020. Potential pesticide exposure during the post-breeding migration of the common toad (*Bufo bufo*) in a vineyard dominated landscape. Science of the Total Environment 706, 134430.
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2. **Leeb C.**, Kolbenschlag, S., Laubscher, A., Adams, E., Brühl, C. A., Theissinger, K., 2020. Avoidance behavior of juvenile common toads (*Bufo bufo*) in response to surface contamination by different pesticides. PLOS ONE 15(11): e0242720.
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3. 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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4. Adams, E., **Leeb, C.**, Brühl, C.A., 2021. Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape. Ecotoxicology 30, 213–223. https://doi.org/10.1007/s10646-020-02335-9

5. **Leeb, C.**, Leschner, M., Busch, F., Brühl, C.A., Sinsch, U., Theissinger, K. 2022. Influence of forest and agricultural landscapes on biometry, age and genetic structure in Palmate Newt (*Lissotriton helveticus*) populations. Salamandra 58(3), 203–217.
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Abstract

In the last decades, it became evident that the world is facing an unprecedented, human-induced global biodiversity crisis with amphibians being one of the most threatened species groups. About 41% of the amphibian species are classified as endangered by the IUCN, but even in amphibian species that are listed as "least concern", population declines can be observed on a local level. With land-use change and agrochemicals (i.e. pesticides), two of the main drivers for this amphibian decline are directly linked to intensive agriculture, which is the dominant landscape type in large parts of Europe. Thus, understanding the situation of amphibians in the agricultural landscape is crucial for conservation measures. In the present thesis, I investigated the effects of viticulture on amphibian populations around Landau in der Pfalz (Germany) in terms of habitat use, pesticide exposure, biometric traits as well as genetic and age structure. From the perspective of amphibians, land-use change means usually the destruction of habitats in agricultural landscapes, which often leads to landscape fragmentation. Thus, I followed the question if also vineyards lead to the fragmentation of the landscape and if pesticides that are frequently used in viticulture have to be considered as a factor too, so if there is a chemical landscape fragmentation. Using telemetry, I could show that common toads (*Bufo bufo*) can be found directly in vineyards, but that they tend to avoid them as habitat. Analysing the genetic structure of common frogs (*Rana temporaria*) revealed that vineyards have to be considered as a barrier for amphibians. To identify if pesticides contribute to the resulting landscape fragmentation, I conducted an arena choice experiment in the laboratory in which I found evidence for an avoidance of pesticide-contaminated soil. Such an avoidance could be one of the underlying reasons for a potential chemical landscape fragmentation. By combining telemetry data with information about pesticide applications from local wine growers, I could show that a large part of the common toads is likely to come in contact with pesticides. Further, I demonstrated that the agricultural landscape, probably due to the application of pesticides, can have negative effects on the reproduction capacity of common toads. By studying palmate newts (*Lissotriton helveticus*) I found that adult newts from agricultural ponds are smaller than those from forest ponds. As I did not find differences in the age structure and growth, these differences might be carry-over effects from earlier life stages. While agricultural ponds might be suitable habitats for adult palmate newts, the potential carry-over effect indicates suboptimal conditions for larvae and/or juveniles. I conclude that the best management measure for sustaining amphibians in the agricultural landscape would be a heterogeneous cultural landscape with a mosaic of different habitat patches that work without or at least a reduced amount of pesticides. Green corridors between populations and different habitats would allow migrating individuals to avoid agricultural and thus pesticide-contaminated areas. This would reduce the pesticide exposure risk of amphibians, while preventing the fragmentation of the landscape and thus the isolation of populations.

Zusammenfassung

In den letzten Jahrzehnten wurde deutlich, dass die Welt mit einer beispiellosen, vom Menschen verursachten Biodiversitätskrise konfrontiert ist. Eine der am stärksten bedrohten Artengruppen stellen dabei die Amphibien dar, so gelten laut IUCN 41% der Amphibienarten als gefährdet. Jedoch können selbst bei Arten die als "least concern" klassifiziert sind Populationsrückgänge auf lokaler Ebene beobachtet werden. Mit einer veränderten Landnutzung und dem Einsatz von Pestiziden sind zwei der Hauptursachen für diese Gefährdung direkt mit intensiver Landwirtschaft verbunden. Daher ist das Wissen um die Situation von Amphibien in der Agrarlandschaft von entscheidender Bedeutung für Schutzmaßnahmen. In der vorliegenden Arbeit wurden Amphibienpopulationen in der durch Weinbau geprägten Gegend um Landau in der Pfalz (Deutschland) im Hinblick auf Lebensraumnutzung, Pestizidexposition, biometrische Merkmale sowie der Alters- und genetischer Populationsstruktur untersucht. Da Agrarflächen oft zur Fragmentierung von Landschaften führen, ging ich der Frage nach ob dies auch auf Weinberge zutrifft und ob eingesetzte Pestizide zur Fragmentierung beitragen, ob also eine chemische Landschaftsfragmentierung vorliegt. Durch die Telemetrierung von Erdkröten (*Bufo bufo*) konnte ich zeigen, dass diese Art direkt in Weinbergen gefunden werden kann, diese aber generell eher meidet. Die Analyse der genetischen Struktur von Grasfröschen (*Rana temporaria*) ergab, dass Weinberge als Barriere für Amphibien anzusehen sind. Um herauszufinden, ob Pestizide zu der daraus resultierenden Landschaftsfragmentierung beitragen, führte ich einen Wahlversuch im Labor durch, bei dem ich ein Vermeidungsverhalten gegenüber kontaminierten Böden fand, was zu einer chemischen Landschaftsfragmentierung führen könnte. Durch die Kombination von Telemetriedaten mit Daten über Pestizidanwendungen von lokalen Winzern konnte ich zeigen, dass ein großer Teil der Erdkröten mit Pestiziden in Kontakt kommt. Außerdem konnte gezeigt werden, dass sich die Agrarlandschaft und hier wahrscheinlich Pestizide negativ auf die Fortpflanzungsfähigkeit von Erdkröten auswirkt. Bei der Untersuchung von Fadenmolchen (*Lissotriton helveticus*) stellte ich fest, dass adulte Molche aus Gewässern in der Agrarlandschaft kleiner sind als Individuen aus Gewässern im Wald. Da kein Unterschied in der Altersstruktur festgestellt werden konnte, könnten diese Größenunterschiede auf suboptimale Bedingungen für Larven und/oder Jungtiere hindeuten, wenngleich Gewässer in der Agrarlandschaft geeignete Lebensräume für adulte Teichmolche sein könnten. Ich komme zu dem Schluss, dass die beste Maßnahme zum Schutz von Amphibien in der Agrarlandschaft eine heterogene Kulturlandschaft mit einem Mosaik aus verschiedenen Lebensräumen wäre, die ohne oder zumindest mit weniger Pestiziden auskommt. Grüne Korridore zwischen Populationen und Teillebensräumen würden es wandernden Individuen ermöglichen, landwirtschaftliche und damit pestizidbelastete Flächen zu vermeiden. Dies würde das Risiko der Pestizidexposition von Amphibien verringern und gleichzeitig die Fragmentierung der Landschaft und damit die Isolation von Populationen verhindern.

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1. Introduction

1.1. Amphibians, an overview

Amphibians are a class within vertebrates that developed about 380 million years ago. Today, 8,407 species are known (AmphibiaWeb, 2021), which are divided into three recent orders that are also called Lissamphibia. With 7,426 known species the order Anura (including all frogs and toads) is by far the largest, followed by Caudata (766 salamander and newt species) and Gymnophiona (215 species). Except for Antarctica, amphibians can be found on all continents, with the highest diversity in tropical regions. Europe, especially Central Europe, is considered to be poor in species with only 51 native Anura and 41 Caudata species and Gymnophiona completely absent (Speybroeck et al., 2020). For Germany, 20 native species (14 Anura, 6 Caudata) are known (Rote-Liste-Gremium Amphibien und Reptilien, 2020).

Amphibians derived their name from the Greek term "amphibious", which means "dual life" and refers to the special biphasic life history and habitat requirements of amphibians. The typical life cycle of amphibians includes an aquatic embryotic and larval development, followed by the metamorphosis during which tadpoles develop into froglets. After the aquatic life stage, amphibians spend most of their life in the terrestrial habitat and visit a water body regularly for mating. However, there are countless exceptions from this life cycle, ranging from a fully aquatic life, over terrestrial mating and terrestrial eggs but aquatic larvae, to a life cycle that is completely independent of an aquatic habitat (see Nunes-de-almeida et al. (2021) for a full classification of amphibian reproductive modes). Species with alternative life cycles can also be found in Central Europe, but the typical life cycle is predominant. For some European species like the common frog (*Rana temporaria*), the agile frog (*R. dalmatina*) or the common toad (*Bufo bufo*), the characteristics of the terrestrial habitat are more important than those of the aquatic habitat (Hartel et al., 2008; Loman and Lardner, 2006). Terrestrial habitats can be several kilometres away from a water body, resulting in long pre- or post-breeding migrations. However, most amphibians use the area in a distance of up to 200 m (95% < 664 m) around a breeding pond as terrestrial habitat, but this distance depends on the species and the distribution of required habitat features in the respective landscape (Rittenhouse and Semlitsch, 2007). In temperate regions, frost-proof hiding places are necessary, because as ectothermic species, amphibians cannot regulate their body temperature, and only a few species can survive sub-zero temperatures over longer periods (Storey and Storey, 2017). Amphibians also rely on a moist environment, as they have a permeable skin (Kaufmann and Dohmen, 2016) which is important for water uptake and gas exchange and even allows the uptake of larger molecules (Llewelyn et al., 2016).

Due to their biphasic life cycle, amphibians represent an important link between the aquatic and the terrestrial world and play a crucial role in both food webs. Anuran larvae are herbivorous to omivorous and are important for the decomposition of organic material in aquatic ecosystems. Caudata larvae are predatory and can thus regulate invertebrates, including vectors of parasitic diseases like mosquitoes (Brodman and Dorton, 2006; Dambach, 2020). Amphibian larvae can have very high densities and biomasses in water bodies. Thus, the metamorphosis represents an important flux of matter to the terrestrial habitat. For example, in South Carolina (USA) over 360,000 juveniles of 24 amphibian species with a total biomass of over 1,400 kg emerged from a single wetland during one breeding season (Gibbons et al., 2006). Terrestrial amphibians are predatory, whereby they feed on everything they can swallow, making them important for pest control in some areas (Civantos et al., 2012; Valencia-Aguilar et al., 2013). At the same time, amphibians are important prey for other vertebrates, especially as they can represent one of the highest vertebrate biomasses in some ecosystems. Amphibians also contribute to several ecosystem services (see Hocking and Babbitt (2014) for a review), and the increasing number of amphibian related citizen science projects (e.g. Genet et al., 2008; Sterrett et al., 2019; Wilkinson and Arnell, 2013) indicates their potential to engage the general public for science and conservation efforts.

1.2. Amphibians are endangered

In the last decades, it became evident that the world is facing an unprecedented, human-induced global biodiversity loss (Dirzo and Raven, 2003). Next to climate change, this biodiversity crisis will be the main challenge for humanity in the 21st century with unpredictable consequences for ecosystems and society (Cardinale et al., 2012; Díaz et al., 2006; Hooper et al., 2012). In Europe, species and population declines can be found across all ecosystems and all species groups, including insects (Hallmann et al., 2017), birds (Burns et al., 2021; Donald et al., 2006) and mammals (Temple and Terry, 2009). For amphibians, first records of population declines arose in the 1950s (Pechmann and Wilbur, 1994). Nowadays, numerous studies are documenting this worldwide amphibian crisis (Alford and Richards, 1999; Allentoft and O’Brien, 2010; Beebee and Griffiths, 2005; Blaustein et al., 2011; Collins and Storfer, 2003; Houlahan et al., 2000; Stuart et al., 2004; Wake, 1991). Amphibians are now considered to be more threatened than other species groups (Harfoot et al., 2021; Hoffmann et al., 2010) and 41% of all species are classified as endangered by the IUCN (IUCN, 2021). However, also in species that are not listed as endangered on an international scale, local population declines can be observed. For example, the IUCN status of the common toad (*B. bufo*) is "least concern" (Agasyan et al., 2009), but population declines have been reported on a local level in the entire distribution area (e.g. Bonardi et al., 2011; Carrier and Beebee, 2003; Kyek et al., 2017; Petrovan and Schmidt, 2016). In the Red List of Germany, half of the native species are classified as threatened, but for 17 out of 20 species a decline during the last 50 to 150 years is suspected (Rote-Liste-Gremium

Amphibien und Reptilien, 2020). Some of the main drivers for the global amphibian decline are invasive species, over-exploitation, emerging infectious diseases, climate change, habitat destruction and chemical pollution (Beebee and Griffiths, 2005; Collins and Storfer, 2003; Grant et al., 2016; Hayes et al., 2010). The last two factors are often linked to the intensification of agriculture since the 1950s (Beebee and Griffiths, 2005). Also in a recent study, agriculture was identified as the most prevalent threat for amphibians worldwide, with an especially high impact probability in Europe (Harfoot et al., 2021).

1.3. Amphibians in agricultural landscapes and the effect of pesticides

Today, about 40% of Europe (Eurostat, 2020a) and 48% of Germany (CIA, 2019) are agriculturally used, making agriculture the most dominant landscape type in many regions (Foley et al., 2005). Therefore, agriculture was one of the main drivers for large-scale land use changes during the last centuries (Kaplan et al., 2012). Extensive agricultures are often mosaics of used and unused areas that can contribute to a more diverse landscape and thus support biodiversity (Benton et al., 2003), including amphibians (Boissinot et al., 2019; Hartel et al., 2010; Hartel and von Wehrden, 2013). In contrast, the intensification of agricultural areas during the last decades is generally considered to have negative effects on biodiversity (Dudley and Alexander, 2017; Emmerson et al., 2016; Reidsma et al., 2006). This intensification often causes a simplification of the landscape (Benton et al., 2003) and can include the drainage of wetlands, leading to the loss of reproduction habitats of many amphibian species. For example, Curado et al. (2011) showed that 57% of ponds disappeared between 1975 and 2006 in an agricultural landscape in northern France. Remaining agricultural ponds are usually man-made and often have a technical function (e.g. for storm water retention). Yet, they can still be habitats for pond-breeding amphibian species (Jumeau et al., 2020; Lenhardt et al., 2013). In some cases, agricultural ponds are considered to have the same value for amphibians as more natural habitats (Orchard et al., 2019). Their importance is even more pronounced when amphibian populations are seen at the meta-population level, as these ponds can be stepping stone habitats between more favourable areas in fragmented landscapes. The location of a pond in an agricultural landscape implies, that amphibians can also be found directly in the agricultural landscape during their pre- or post-breeding migration. During this time, amphibians face several risks in the course of agricultural processes such as mowing or mechanical soil tillage, which can result in direct mortality (Humbert et al., 2009; Pfeffer et al., 2011). However, one of the greatest dangers might come from exposure to pesticides that are frequently used in intensive agricultural landscapes.

Each year approximately 360 million kg pesticide formulations are used in agricultural fields in the European Union to control pests, weeds and diseases in order to improve crop yields (Eurostat, 2020). There are several pesticide classes, targeting different pest groups with herbicides (against unwanted plants), fungicides (against pathogenic fungi and oomycetes) and insecticides (against insects) being the three most common classes with in total 327 million kg per year (Eurostat, 2020). In some crop types up to 21 pesticide applications per year can be observed in Central Europe (e.g. apple orchards in Germany; treatment index = 33; Roßberg, 2013). However, only a small part of the applied pesticides reaches their target organism, and the larger part enters the surrounding environment (Pimentel, 1995). As many pesticides do not breakdown rapidly following application, they can accumulate in soil and vegetation. In fact, most agricultural topsoil is contaminated with pesticides (Hvězdová et al., 2018; Silva et al., 2019). Due to spray drift, pesticides can also be found in the vicinity of the actual application site. In some cases 10% of the applied pesticide concentration can be measured in a distance of 3 m to a field (Cusaac et al., 2015; Otto et al., 2015). Ernst et al. (1991) documented pesticide drift over 200 m, and Shunthirasingham et al. (2011) even detected the insecticide endosulfan sulfate in bromeliads in the cloud forests of Costa Rica because of atmospheric transport, kilometres away from the next arable field. Also pesticide exposure of mobile species like insects, which are a common prey for amphibians, leads to a spread of pesticides to areas where they are not applied (Brühl et al., 2021). Due to spray drift and run-off, pesticides can also get into water bodies within or near agricultural fields (Goessens et al., 2022; Reichenberger et al., 2007; Zhang et al., 2018). Thus, amphibians can be exposed to pesticides in both their aquatic and terrestrial life stage.

Estimating the exposure of aquatic amphibian life stages is comparably easy because eggs, larvae or reproducing adults can be reliably detected in the aquatic habitat, and the pesticide concentrations in the water can be measured also over a specific time period. In contrast, such an estimation is more challenging for the terrestrial habitat, as it requires detailed information about a species' terrestrial habitat use as well as information about the number and timing of pesticide applications. Although several studies showed that amphibians can be found in agricultural fields (Knutson et al., 2004; Müller et al., 2013; Schweizer, 2014), only a few took the next step and combined these data with information about actual pesticide applications to demonstrate a spatial-temporal overlap of pesticides and amphibians in the field. Berger et al. (2013) used drift fences to study the migration of four temperate amphibian species and estimated the risk of exposure to the herbicide glyphosate. They also analysed the spatial-temporal overlap with mineral fertiliser, another group of agricultural chemicals (Berger et al., 2012). Lenhardt et al. (2015) expanded the analysis of Berger et al. (2013) by taking into account applications of other pesticides and the interception by crops. All three mentioned studies documented a high spatial-temporal overlap, but it has to be pointed out that they are based on the same three-year data set from only one study site in northeast Germany. Although some studies provide evidence for previous pesticide exposure by detecting pesticides in tissue of amphibians (e.g. Smalling et al., 2015) and some directly linked them to agricultural habitat use (e.g. Swanson et al., 2018), the actual knowledge on the question whether amphibians are present in agricultural fields during pesticide applications is still limited.

Exposure to pesticides can have negative effects on amphibians during both the aquatic and the terrestrial life stage. In larvae it can result in impairments in the behaviour like abnormal swimming, decreased activity and decreased feeding (Agostini et al., 2020; Sievers et al., 2019), as well as in deformations (Baier et al., 2016), damages of the DNA (Gonçalves et al., 2017), reduced sizes at metamorphosis (Baker et al., 2013) and reduced survival rates (Agostini et al., 2020; Baker et al., 2013). While effects during the aquatic life stages are studied frequently, there is a lack of studies focusing on pesticide exposure during the terrestrial life stages (Brühl et al., 2011). However, negative effects like altered biochemical processes in the liver (Van Meter et al., 2018), a decreased brain cholinesterase activity (Henson-Ramsey et al., 2008), alterations in the hepatic tissue and erythrocyte nuclear abnormalities (Franco-Belussi et al., 2016), increased fatigue (Mitchkash et al., 2014) or malformations (Guerra and Aráoz, 2016) had been documented. Less is known about the effects on the behaviour of terrestrial or adult amphibians. Several studies investigated if there is an avoidance behaviour against contaminated water bodies (Takahashi, 2007; Vonesh and Buck, 2007; Wagner and Lötters, 2013) and surfaces (Gertzog et al., 2011; Ortiz-Santaliestra et al., 2005; Storrs Méndez et al., 2009), but results are partly contradictory. Adams et al. (2021a) showed that exposure to contaminated soil affects the locomotor activity and the feeding behaviour of juvenile *R. temporaria*. What is even more critical is that they further observed direct mortality of up to 60% of the test animals. High mortality rates, in some cases of up to 100%, can also be found when amphibians are directly oversprayed with pesticides at field rates in the laboratory (Belden et al., 2010; Brühl et al., 2013; Relyea, 2005). Beside possible direct effects on amphibians, indirect effects, e.g. effects of the pesticide on the food web and thus on the availability of prey, are probable (Brühl and Zaller, 2019).

The effects mentioned do not only affect the individual, but also populations and meta-populations. Impairments in the development and mortality in larvae can lead to smaller and fewer metamorphs. Juveniles play an important role in the dispersal in many amphibian species (Cushman, 2006) and the dispersal ability of an individual can often be linked to body size (Phillips et al., 2006; Trochet et al., 2019, 2016b). Thus, this might directly affect the migration and therefore the gene flow between populations, which can then lead to reduced fitness of a population (Allentoft and O'Brien, 2010). Gene flow is also restricted when terrestrial amphibians die after contact with pesticides. In cases where an individual avoids a contaminated area, its individual risk might be reduced, but when green corridors in intensive agricultural landscapes are lacking, this might lead to a reduced gene flow too. Some studies investigated the effects of agricultural landscape on the genetic structure or gene flow between amphibian populations (e.g. Costanzi et al., 2018; Frei et al., 2016; Johansson et al., 2004), but the landscape fragmentation due to pesticides, i.e. chemical landscape fragmentation, has largely been neglected so far.

2. Objectives of the thesis

The overall goal of this thesis was to contribute to the assessment of amphibians in agricultural landscapes and to investigate a potential (chemical) landscape fragmentation. I focused on three amphibian species (*Bufo bufo*, *Rana temporaria* and *Lissotriton helveticus*) in a vineyard dominated landscape (Fig. 1). The study species were selected based on their abundance in the study area around Landau in der Pfalz (southern Rhineland-Palatinate, Germany). In addition, they represent species with different migration capacities, ranging from hundreds of meters (*L. helveticus*) to several kilometres (*B. bufo*). Different aspects of the overall goal were addressed in studies ranging from laboratory over semi-field to field studies and are presented in five chapters of this thesis.

Chapter 1

*Potential pesticide exposure during the post-breeding migration of the common toad (*Bufo bufo*) in a vineyard dominated landscape.*

In chapter 1 we used telemetry to track common toads daily during their post-breeding migration to answer one of the most basic questions in the context of amphibians in viticultural landscapes: Do common toads use vineyards as terrestrial habitat or do they avoid them? Additionally, we combined the data on the post-breeding migration with real pesticide application data from local wine growers. This allowed the identification of a potential spatial-temporal overlap of pesticide applications with the terrestrial activity phase of common toads, as well as the estimation of the pesticide exposure risk of the population.

Chapter 2

*Avoidance behavior of juvenile common toads (*Bufo bufo*) in response to surface contamination by different pesticides.*

For amphibians a permanent crop such as vineyards might be a more suitable habitat than other types of agriculture. Thus, the question arises whether avoidance of vineyards could - at least partly - be the result of avoidance of pesticide contaminations, which would be a strong indicator for chemical landscape fragmentation. To answer this question, we performed a laboratory experiment, in which juvenile common toads were placed in test arenas, of which half was oversprayed with one of seven tested pesticides in rates of 10% or 100% of the maximum recommended field rate. Five fungicides, one herbicide and one insecticide used in viticulture were tested. We then filmed the toads for 24 h and used tracking software to calculate the proportion of time a toad spent on the contaminated side to identify a potential avoidance behaviour of the toads towards pesticides contaminations. As video analysis with tracking software is not always feasible, we further tested the effect of changing the sampling interval for manual data analyses.

Chapter 3

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

In chapter 3 we analysed microsatellite data to assess the gene flow between common frog (*Rana temporaria*) populations as an indirect detection method for landscape fragmentation. Landscape genetic methods were used to identify landscape elements that explain the observed genetic differentiation and thus to answer the question whether vineyards must be considered as a barrier for amphibians.

Chapter 4

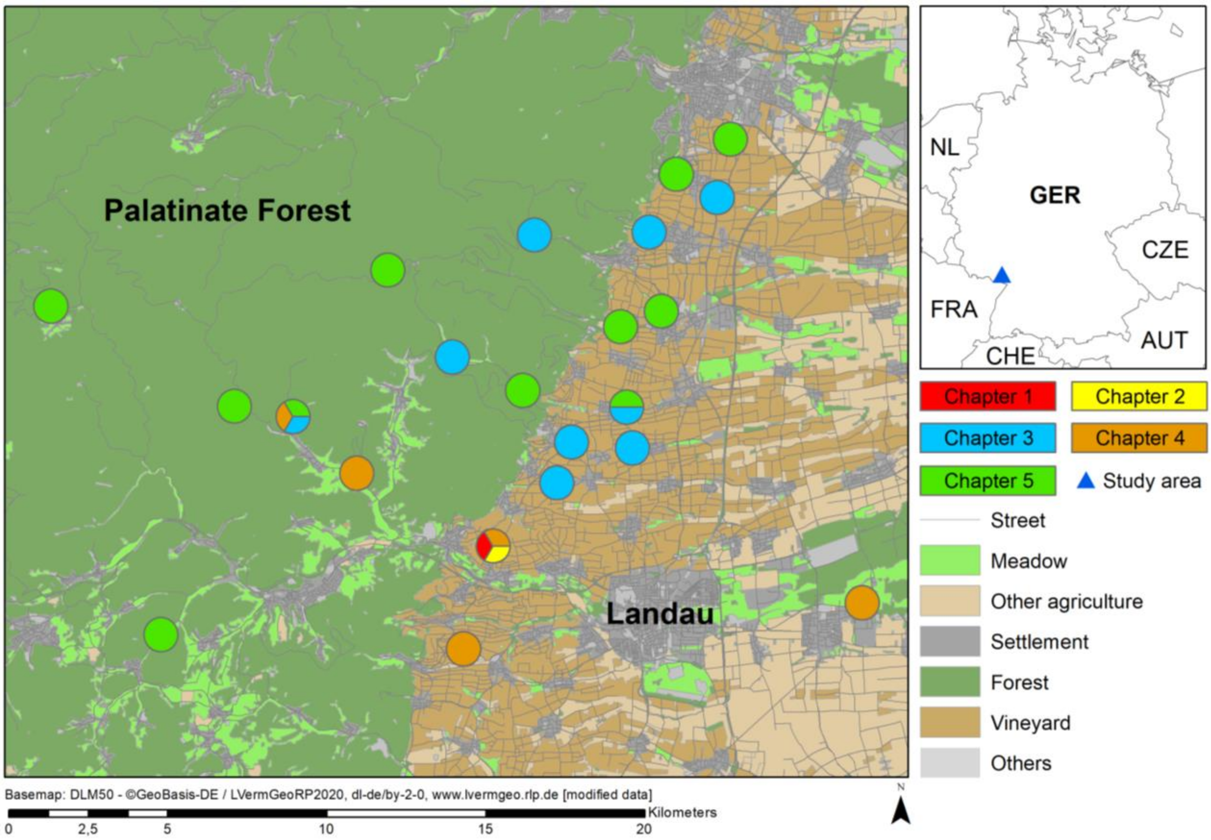
*Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape.*

Besides (chemical) landscape fragmentation also the number of individuals that migrate between populations affects the gene flow between them. In amphibians, juveniles play an important role in dispersal. Impairments in the reproduction like a decreased fecundity (i.e. fewer eggs) and/or a lower fertilization rate (i.e. fewer hatched tadpoles) as well as a lower survival rate and a reduced size of tadpoles would thus contribute to a reduced gene flow between populations. In a semi-field study, we investigated the reproductive capacity of common toads from five breeding ponds with different pesticide contamination levels and different proportions of agricultural areas around the pond.

Chapter 5

*Influence of forest and agricultural landscapes on biometry, age and genetic structure in palmate newt (*Lissotriton helveticus*) populations.*

In chapter 5 we investigated palmate newt (*Lissotriton helveticus*) populations from an intensive wine-growing region and compared them with populations located in nearby forested areas in terms of biometric traits, age and genetic structure. Differences in biometric traits and age structure would indicate negative effects of the habitat (forest/agriculture) on amphibian populations, while differences in the genetic structure would indicate a reduced gene flow between populations and therefore a fragmentation of the landscape.



Basemap: DLM50 - ©GeoBasis-DE / LVermGeoRP2020, dl-de/by-2-0, www.lvermgeo.rlp.de [modified data]

Fig. 1: Overview of the study area in the greater area of Landau in der Pfalz (southern Rhineland-Palatinate, Germany). It is characterised by the Palatinate Forest in the west and agricultures, dominated by vineyards, in the east. Circle colour refers to amphibian populations used in the studies presented in chapters 1-5. Amphibian populations in chapter 1, 2 and 4 = *Bufo bufo*, chapter 3 = *Rana temporaria*, chapter 5 = *Lissotriton helveticus*.

Chapter 1

Potential pesticide exposure during the post-breeding migration of the common toad (*Bufo bufo*) in a vineyard dominated landscape

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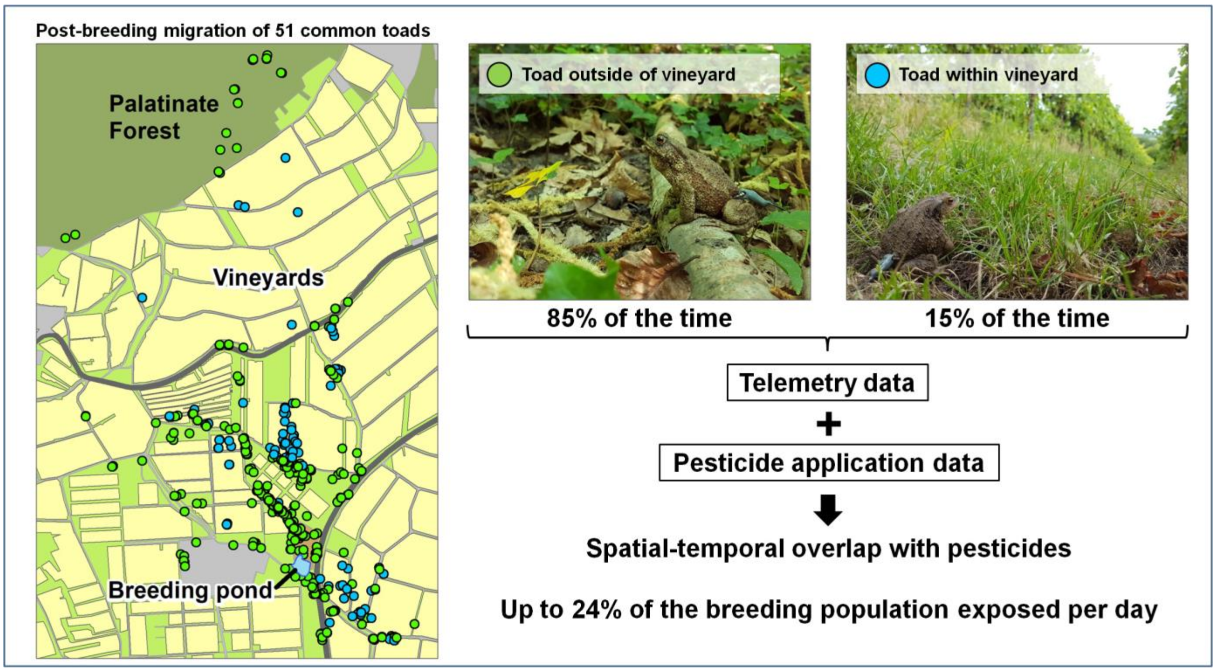
Highlights

- We used telemetry to track common toads in an agricultural landscape
- Toads are using vineyards as habitats during their post-breeding migration
- Comparing used and available habitats shows that toads tend to avoid vineyards
- Up to 24% of the breeding population are exposed to pesticides per day
- A heterogeneous cultural landscape is vital for the conservation of amphibian populations

Graphical abstract



Abstract

Two important drivers of the global amphibian decline are habitat destruction due to an intensification of farming and a related increase of pesticide applications. Recent studies have shown that there might be an underestimated risk of pesticides on terrestrial amphibians. However, there are too few data on the terrestrial habitat use of amphibians in agricultural landscapes to estimate the exposure risk. To fill this knowledge gap, we used telemetry to investigate the post-breeding migration of 51 common toads (*Bufo bufo*) from a breeding pond in a vineyard-dominated landscape in Southern Palatinate (Germany). We expected most toads to migrate to the nearby Palatinate Forest as a terrestrial habitat. However, only four individuals reached the forest, suggesting that a part of the population is inhabiting the agricultural landscape over large parts of the year. Individuals were also found directly in the vineyards (15% of all relocations), but 23% less often than expected from a random choice and therefore tend to avoid vineyards as terrestrial habitat. To estimate a possible spatial-temporal overlap of toad migration and pesticide application, we combined telemetry data with information about pesticide applications from local wine growers. Seven individuals had a high probability (> 75%) of being directly exposed to a pesticide application. Taking spray drift and the half-life values of applied pesticides into account, the number of toads potentially exposed raised to 15 individuals. We estimated that, on a single day up to 24% of the whole breeding population came in contact with pesticides, resulting in a high overall exposure risk. Pesticides can have negative effects on amphibians, and toads try to avoid vineyards as habitats. Therefore, we conclude that a heterogeneous cultural landscape, with buffer strips around ponds, uncultivated patches and migration corridors, might be the best management measure for sustaining amphibians in the agricultural landscape.

Keywords

Telemetry, avoidance behavior, fungicide, viticulture, amphibian, habitat selection

Introduction

Amphibian populations are declining on a global scale (Alford and Richards, 1999; Houlahan et al., 2000; Stuart et al., 2004) and today 40% of all amphibian species are considered endangered (IUCN, 2018). Some of the main drivers for this decline are climate change, invasive species, habitat destruction and chemical pollutions (Beebee and Griffiths, 2005; Collins and Storfer, 2003; Grant et al., 2016; Hayes et al., 2010). The last two factors are often linked to the intensification of agriculture since the 1950s (Beebee and Griffiths, 2005).Agriculture is representing the dominant landscape type in many areas of the world (Foley, 2005) and today about 40% of the area of the European Union (Eurostat, 2019) and 48% of Germany (CIA, 2019) is agriculturally used. The agricultural landscape of large parts of Southern Palatinate (Germany) is dominated by viticulture (Lenhardt et al., 2013): wine is cultivated by about 2,300 wine growers on an area of 234 km² (Statistisches Landesamt Rheinland-Pfalz, 2018). In this region, rain retention ponds serve as important habitats for several pond-breeding amphibian species (Lenhardt et al., 2013).

Although amphibians are usually associated with aquatic habitats, most temperate, biphasic amphibian species also depend on a suitable terrestrial habitat. For some European species like the common frog (*Rana temporaria*), the agile frog (*R. dalmatina*) or the common toad (*Bufo bufo*), the characteristics of the terrestrial habitat are even more important than those of the pond (Hartel et al., 2008; Loman and Lardner, 2006). As terrestrial habitat, vineyards might be more suitable for amphibians than other types of arable land, as wine is a permanent crop with greened rows and shade during the summer. However, wine is usually cultivated in dry and warm areas, resulting in suboptimal microclimates for most temperate amphibians. Viticulture is also a very intensive form of agriculture, requiring mechanical practices such as tillage and mowing or the removal of leaves on the vine plants. Additionally, there are on average 9.5 pesticide applications between March and mid-August in Germany, where mainly fungicides (average 8.8 applications), often as a mixture of several products (average 1.6 products per application), are preventively used to protect the plants primarily from powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*) (Roßberg and Ipach, 2015). Pesticides are often applied shortly before or after a precipitation event (Lenhardt et al., 2013). The high number of pesticide applications involving tractors and other machinery might induce an additional disturbance of amphibians and result in a further devaluation of vineyards as terrestrial habitat.

Studies on the detailed habitat use of amphibians in viticulture are lacking, but Tanadini et al. (2012) showed that vineyard-dominated and environment-friendly managed landscapes in Switzerland can be habitats for fire salamanders (*Salamandra salamandra*), a species with a mainly terrestrial life history. In general, within agricultural landscapes man-made ponds often serve as important aquatic habitats for pond-breeding amphibian species (Beja and Alcazar, 2003; Berger et al., 2011; Knutson et al., 2004; Lenhardt et al., 2013). Consequently, amphibians can be found within agricultural fields during their post-breeding migration (Berger et al., 2013; Kovar et al., 2009; Miaud and Sanuy, 2005; Schweizer, 2014), especially when migration corridors are lacking. Depending on the time they spend in a field during the post-breeding migration, they are potentially exposed to dangers linked to agricultural practices like mechanical tillage or pesticide applications.

Several studies demonstrated that pesticides can have a negative effect on amphibians (see reviews in Brühl et al. (2011), Fryday and Thompson (2012) and Mann et al. (2009)), but only a few investigated the effects of pesticides on terrestrial life stages. Compared to other vertebrates terrestrial amphibians have a highly permeable skin (Kaufmann and Dohmen, 2016) that has an important role in water regulation (Wells, 2007), but also facilitates the dermal uptake of pesticides (Storrs Méndez et al., 2009; Van Meter et al., 2014). Pesticide uptake is highest when individuals are directly oversprayed with pesticides (Van Meter et al., 2015), and high mortality rates of up to 100% of tested individuals can be observed at field application rates (Belden et al., 2010; Brühl et al., 2013; R. A. Relyea, 2005). Moreover, many pesticides do not breakdown rapidly following application but remain on vegetation and soil (Hvězdová et al., 2018; Silva et al., 2019). Thus, amphibians might come in contact with pesticides, even if they are not directly oversprayed. This could lead to a dermal pesticide uptake (Storrs Méndez et al., 2009; Van Meter et al., 2015) and a chronic exposure of amphibians in agricultural areas. This might be especially true as terrestrial amphibians are probably unable to detect and therefore avoid contaminants on natural soils (Hatch et al., 2001; Storrs Méndez et al., 2009). Such an uptake might not result in direct mortality, but in an accumulation of pesticides and sub-lethal effects (Franco-Belussi et al., 2016; Henson-Ramsey et al., 2008; Mitchkash et al., 2014; Van Meter et al., 2018). Due to spray drift even individuals that are not directly in agricultural fields might be exposed to pesticides. In a distance of 3 m to the next agricultural field, concentrations of about 10% of the applied concentration can be found (Cusaac et al., 2015; Otto et al., 2015). For some pesticides this is a rate that can lead to mortality in amphibians (Brühl et al., 2013). In general, detailed studies on the spatial-temporal overlap of amphibian migration and pesticide application are still scarce (but see Berger et al. (2013) and Lenhardt et al. (2014)), and to our knowledge investigations on the pesticide exposure risk of amphibians in vineyards do not exist.

In the present study we used telemetry to investigate the amphibian post-breeding migration and habitat use in a vineyard dominated landscape. As model organism we used the common toad (*B. bufo*), because it is one of the most common amphibian species in the study area (Lenhardt et al., 2013). The studied population consists of several hundred individuals breeding in a rain retention pond. The area around this pond is dominated by vineyards, but also shrubberies and grassland can be found. In a distance of 900 m to the pond the Palatinate Forest begins, one of the biggest contiguous blocks of forests in Central Europe. As the forest is within the migration capacity of common toads (Kovar et al., 2009; Sinsch, 1988; Sztatecsny and Schabetsberger, 2005), and the species is often associated with forest habitats (Hartel et al., 2008; Romero and Real, 1996; Salazar et al., 2016), we expected that most toads migrate to the Palatinate Forest after breeding. However, during migration *B. bufo* might cross vineyards and use those as temporary habitat, where they could be exposed to pesticides. We used telemetry data from 51 individuals and obtained data on pesticide applications in vineyards to ask if a) common toads are using vineyards as habitat during their post-breeding migration or if they are avoiding them, and if b) there is a spatial-temporal overlap with pesticides and therefore an exposure of the common toad population.

Material and methods

Study species & study site

The common toad, *Bufo bufo* (Linnaeus, 1758), is a medium sized anuran species (males up to 89 mm and 63 g; females up to 111 mm and 130 g; Günther and Geiger (2009)) and one of the most common amphibians in Europe (Sillero et al., 2014). It inhabits a wide range of habitats including agricultural landscapes. Although living mainly terrestrial, it depends on an aquatic habitat for reproduction, like most temperate amphibian species. In Germany, mating usually takes place after hibernation during a short period in March and April (Günther and Geiger, 2009). Aquatic and terrestrial habitats can be far apart, so annual (mass) migrations from a suitable hibernation site towards the breeding pond and back to the terrestrial habitat over up to three kilometers can be observed (Günther and Geiger, 2009). Although the common toad is not considered endangered on an international ("least concern" by the IUCN; Agasyan et al., 2009) or national ("least concern" by the red list Germany; Kühnel et al., 2009) scale, population declines have been reported on a local level in the entire distribution area (e.g. Beebee and Carrier (2003), Bonardi et al. (2011), Kyek et al. (2017) or Petrovan and Schmidt (2016)).

We investigated the post-breeding migration of a *B. bufo* population in Siebeldingen (Rhineland-Palatinate, Germany). Here, a permanent rain retention pond (49.218368 N, 8.049538 E (WSG84), 196 m asl), surrounded by a vegetative buffer strip, is an important breeding reservoir with several hundred breeding individuals each year. The landscape around the pond is dominated by vineyards (Fig. 1), but also forests, shrubberies and uncultivated land can be found. Vine is cultivated conventionally as well as organically by several wine growers in small, neighboring parcels. The proportion of organic viticulture in our study area is not known, but it is higher than in whole Palatinate (about 10%; Statistisches Landesamt Rheinland-Pfalz, 2018). Beside agricultural roads, two roads are close to the pond. One of these roads is located between the breeding pond (distance pond and road about 500 m) and the Palatinate Forest (distance to pond about 900 m). Here, a local nature conservation group uses an amphibian fence to reduce road mortality of the common toads during their spring migration each March and April. Between 2008 and 2018 the mean annual temperature in the study area was 11.1°C and the average annual precipitation was 684 mm (data from the weather station "Siebeldingen 88 / SBD" in a distance of about 200 m to the pond; Weather station Siebeldingen, (2019)).

Sampling of toads and preparation for telemetry

Common toads were captured in the pond surroundings after their reproduction in March 2017. Toads were measured with a ruler to the nearest mm (snout-vent-length; SVL), weighed with a digital scale (G & G TS-WB+G 300g/0,01g, G&G GmbH, Germany) to the nearest 0.1 g and sexed based on the presence of nuptial pads (Günther and Geiger, 2009). We aimed to have a balanced sex ratio for telemetry. A photo of the dorsal and the ventral side was taken to recognize a toad in case that the transmitter was shed. Captured toads were kept in 90 L buckets equipped with moist soil and leaves for hiding until the evening. After sunset a BD-2 radio transmitter with a whip-antenna (2.1 g, lifespan of 20 or 24 weeks; Holohil Systems Ltd, Carp Ontario, Canada) was attached externally with an aluminum chain belt around the toad's waist (mounting belt = 0.9 g). We used 20 transmitters with individual frequencies. The chain was covered with brown Plasti-dip (PLASTI DIP International Inc., Blaine, Minnesota, USA) to reduce lateral abrasion and reflections of the metal and therefore decreasing the probability of predation (Indermaur et al., 2008). This mounting technique was successfully used in several amphibian telemetry studies (e.g. Daversa et al. (2012), Frei et al. (2016) or Indermaur et al. (2008)). Following the recommendation of Richards et al. (1994), that the weight of a transmitter should not exceed 10% of the body, only individuals with more than 30 g were used. Toads were released at the place of capture after handling.

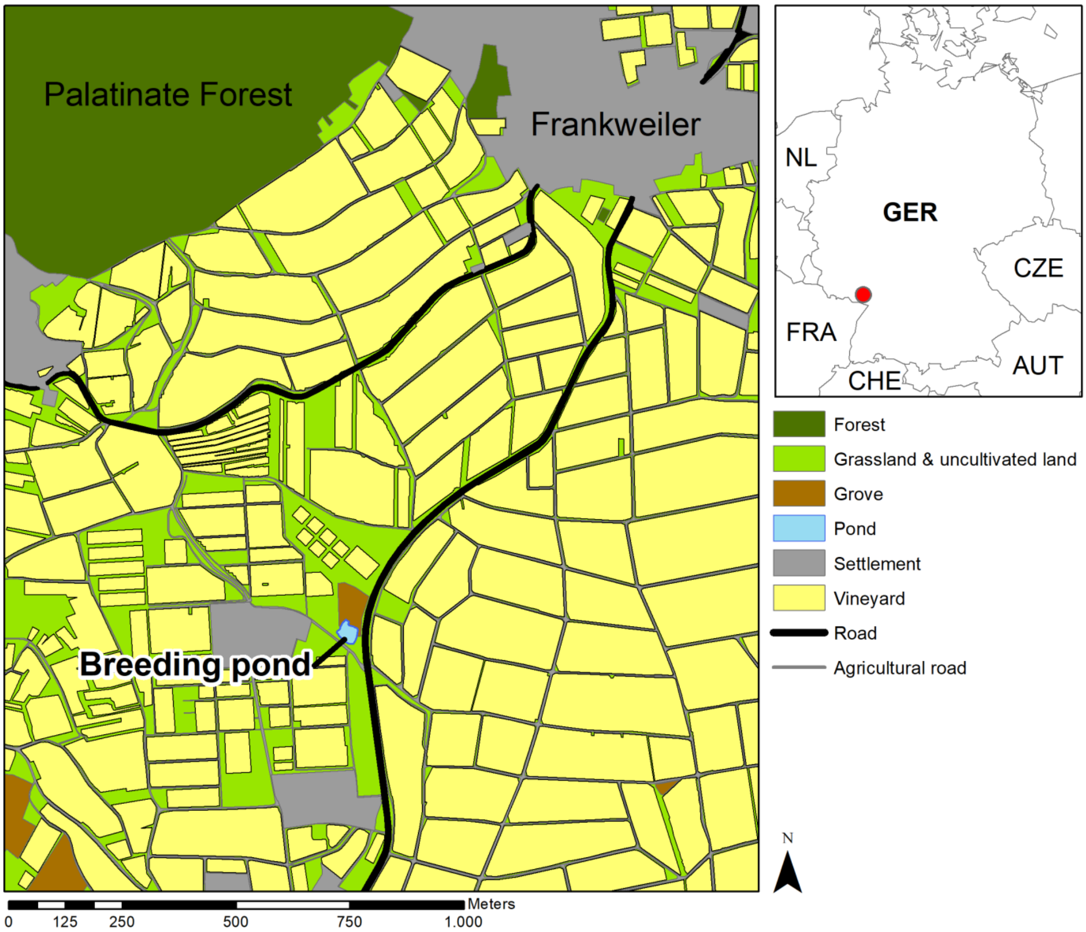


Fig. 1: Map of study area. The distance to the Palatinate Forest is about 900 m. The area is dominated by vineyards.

Telemetry and data collection

Between the 18th of March and the 7th of September 2017 we located toads on a daily basis during day time using a telemetry receiver (R-1000, Communication Specialists Inc., CA, USA) with a Yagi-antenna via homing-in with an accuracy of 30 cm and, if possible, a visual verification of their presence. Every movement exceeding 30 cm was recorded. For movements below 30 m the exact position was determined using a compass and a 20 m measuring tape. For longer distances a GPS (Garmin eTrex Summit, International Inc., Olathe, KS, USA) was used. As this device has an accuracy of > 4 m, we recorded the position three times with a time lag of several minutes and averaged the coordinates. When a precise relocation was not possible without the destruction of the surrounding habitat (e.g. when the toad was sitting in a dense bramble bush), endangering the toad or when it was sitting on private property, we used triangulation of the signal from different locations to estimate the toads´ position. On each new position we estimated the proportion of the toad that was covered (e.g. with vegetation or soil), seen from about 30 cm above. We also noted whether or not a toad was sitting within a vineyard. If so, we measured the distance to the next vine row and the distance between two rows with a measuring tape; if not, we measured the distance to the next vineyard. For distances over 30 m or when measuring in the field was not possible the distance was measured in a GIS. For this purpose, a detailed vector map of all vineyards in the study area and the breeding pond was created based on high resolution orthophotos (Luftbild RP Basisdienst; photos taken on 15th of August 2016; resolution of 40 cm per pixel). Based on this map, we also measured the distance to the pond. We also noted every change on the toads´ position and human activity (e.g. pesticide application or mechanical soil tillage) that might had influenced the toads´ behavior. The health of the study animals, their weight and the fit of the belt was checked periodically. The transmitter was removed in cases of injuries, at the end of the study period or when the toad stayed in the Palatine Forest for over one month. When a toad was found dead or only the transmitter was found, all relocations since the last visual encounter or the last detected movement were excluded from the data set. Then, we reused the transmitter on a new toad collected in the pond surroundings, whereby no toad was tracked twice. To examine if toads use the forest for hibernation we tracked six additional toads during October and November 2017 on a weekly basis.

Ethics statement

The study was approved by the ”Struktur- und Genehmigungsdirektion Süd Referat 42 - Obere Naturschutzbehörde” (Neustadt an der Weinstraße, Germany; approval number: 42/553-254/457(17)). If necessary, the permission to enter private areas was given by the landowners.

Data analysis

All spatial data were handled in ArcGIS 10.4.1 (ESRI, Redlands, California) with the help of python-scripts (python 2.7.10). Statistical analyses were performed in R (R Core Team, 2017). For GLMMs the "mixed" function in the R package afex (Singmann et al., 2018) was used, whereby p values for all fixed factors were calculated via likelihood ratio tests. For all statistical tests the criterion for significance was 0.05.

Post-breeding migration behavior

For each toad we calculated the mean Euclidean distance between the positions before and after a movement (d_{mean}), the total distance moved (d_{total}) and the mean and maximum distance between the pond and the position of a toad (d_{pond_mean}, d_{pond_max}). Moreover, we calculated an activity index (*AI*), defined as the number of detected movements divided by the number of days tracked. For individuals with at least three different positions the area of the home range (95% minimum convex polygon: mcp_{95}) was calculated using the function "mcp" in the R-package adehabitatHR (Calenge, 2006). We used linear models to investigate if sex, body condition (scaled mass index, SMI; see Peig and Green (2009)) or the tracking time had an influence on d_{total}, d_{mean}, d_{max}, AI and mcp_{95}. We tested dependent variables for normal distribution with a Shapiro-Wilk test and used log and square root transformations to obtain normally distributed data if necessary.

Vineyards as habitat

To analyze whether toads actively avoid vineyards we performed a design III resource selection study (Thomas and Taylor, 1990), where for each single individual an available and used resource (i.e. habitat) is compared. Therefore, we considered each movement as a vector $\overrightarrow{AB}$ and used the selected habitat at position B (vineyard yes/no) as used resource. The same binary classification was used to calculate the proportion of available resources. For this calculation we assumed that the toads' movements were limited to their individual migration capacity that depends on the day. We thus calculated the available habitat for a distance of $\overrightarrow{AB}$ + 10% around A ($H_{available}$). Further, we expected that toads followed a target-orientated movement. Hence, we limited $H_{available}$ to an area of ± 45° to the direction of $\overrightarrow{AB}$. To determine the proportion of vineyards in $H_{available}$ the vector map of the vineyards in the study area was used. To compensate the effect of possible inaccuracies during the mapping all vineyard-polygons were buffered by minus two meters (average distance between two wine rows; Roßberg and Ipach (2015)). We calculated the available and used habitat for each individual over all observed movements and compared them with a two sided Wilcoxon signed-rank test. Movements where $H_{available}$ was completely within or outside of vineyards were excluded, as we expected that the toad didn't have the possibility to reach a vineyard or another habitat, respectively, and therefore no free choice on this day. We also excluded movements (5.6 %) that were limited, e.g. because they took place in a sewer.

A binomial regression model was used to analyze if the probability to be detected in a vineyard at least once was the result of the number of days the individual was tracked. To determine whether the overall probability that a toad was using a vineyard as habitat increased during the year, we used a binomial generalized linear mixed model (GLMM) with the habitat type (vineyard - yes/no) of all positions as response variable and the date of the relocation as predictor. To take the individual behavior from each toad into account we used the individual as random factor. Because individuals, which were tracked only for some days, cannot provide information on whether or not the probability to be detected in a vineyard increased over time, we excluded all individuals that were tracked less than 86 days (i.e. the half of the study time) for this GLMM. To test if the probability for a toads´ movement is higher in a vineyard compared to other habitats, we used a binomial GLMM. A significantly higher probability to change the position would indicate that toads are spending less time in this habitat. As movements might be induced by other factors besides the habitat, also a possible disturbance (e.g. tillage), the handling of a toad (e.g. checking the fit of the transmitter), and data on weather conditions (air temperature, humidity and precipitation; see Table A in Appendix A) were used as fixed factors and the individual again as random factor. Movements directly after the attachment of the transmitter were removed from the dataset.

We further analyzed if toads chose a random position within vineyards or if they preferred to sit right under a vine row. Therefore, we used a two sided Wilcoxon signed-rank test to compare the distance from a toads position to the next vine row with one quarter of the width between two vine rows (range: 1.7 m to 3.1 m) in that vineyard. One quarter of the width would be the average expected value if toads are choosing a random location when sitting in a vineyard.

Pesticide applications and spatial-temporal overlap

We obtained pesticide application data from ten conventional wine growers located < 10 km around the study site. We assumed that these application data are representative for our whole study area, even if a part of the area is managed organically. We calculated the probability (p) that a vineyard was treated with pesticides on the day d as

$$p_d = \sum_{F=1}^{F=n} \frac{a_{F,d}}{n}$$

where $a_{F,d}$ is the percentage of the vineyards of the wine grower F that was treated on day d, and n is the number of all wine growers that reported pesticide application data (n = 10). When a wine grower reported that an application of all vineyards was spread over two or more days, we assumed that the same percentage of its vineyards was treated on each day.

We combined p_d with presence/absence data of toads in vineyards to calculate the probability to come in contact with a pesticide at least once for each individual we tracked as

$$exp_p = 1 - \prod_{d_{first}}^{d_{last}} (1 - v_d * p_d)$$

where d_{first} is the day of the first and d_{last} the day of the last successful relocation of the individual and v_d is the binary information whether the toad was found within a vineyard on day d.

Further we estimated the number of days with expected coincidences for each individual as

$$exp_n = \sum_{d_{first}}^{d_{last}} v_d * p_d$$

Finally, we calculated the percentage of the whole (breeding) population that can be expected to come in contact with pesticides for each day as

$$pop_d = \frac{nv_d}{nt_d} * p_d * 100 * \ Pleft_d$$

where nvd is the number of individuals that were found in a vineyard and nt_d is the total number of individuals that were tracked on d. The term $Pleft_d$ describes the part of the population that had not reached the forest on day d and could therefore come in contact with pesticides. To calculate $Pleft_d$ we assumed that all individuals were migrating to the Palatinate Forest or another suitable habitat as long-term habitat or hibernation and thus left the agricultural area over the year. As we observed different arrival times in the forest (see 3.1), we assumed that this migration is a linear process over 249 days between the 18th of March (first observed mating) and the 22th of November 2017 (first sub-zero temperatures; weather station "Siebeldingen 88 / SBD"). This results in a decrease of $Pleft$ by 0.4% per day.

As an example from our data, on the 27th of July (=131 days after the first breeding) 6 (= nv_d) of 9 (= nt_d) tracked individuals were found within a vineyard. On this day the probability that a vineyard was treated with pesticides was 28% (p_d= 0.28). This resulted in a probability that an individual came in contact with a pesticide of 6/9 * 28% = 19%. We assume that on the 27th of July already 131*0.4% = 52.4% of the population had reached the forest, leading to the assumption that 47.6% of the population was still present within the agricultural area. So we can expect that on this single day 9% (= 19% of 47.6%) of the whole breeding population came potentially in contact with a pesticide.

Because pesticides do not break down immediately following application, we took the persistence of pesticides into account and recalculated exp_p, exp_n and pop_d. Therefore, we used the half life values of the pesticides (DT_{50}) and grouped them according to Lenhardt et al. (2014): A pesticide with a DT_{50} < 1 day was expected to have a persistence of one day, a DT_{50} from 1-50 days a persistence of three days and a persistence of 14 days was assumed for pesticides with a DT_{50} > 50 days. In case pesticides had been applied in mixtures of several formulations or a formulation had more than one active ingredient, we considered the active ingredient with the highest DT_{50}. For pesticides where the DT_{50} value was not known, we assumed a persistence of three days (median value of all formulations). Toads sitting near vineyards might come in contact with pesticides due to spray drift. Within a distance of 3 m to the next vineyard pesticide might be found in concentrations that could harm amphibians. Consequently, all toads in a distance of < 3 m to the next vineyard were defined as within a vineyard when recalculating exp_p, exp_n and pop_d.

Results

Post-breeding migration behavior

Between the 18th of March and 7th of September 2017 51 toads (25 males, 26 females) were tracked (Appendix B), whereby only one individual could be tracked over the whole time (173 days). The most common reasons why the tracking ended was that the transmitter was shed (19 times) or removed because of skin injuries caused by the transmitter (eight times) and because the individual died because of predation (eleven times; see Appendix B for details). Six individuals shed the transmitter or died before the first relocation and were therefore excluded from analysis. The other individuals were relocated between one and 173 times (mean = 47 ± 40 days). In total, we recorded 517 positions changes over 30 cm, 11.5 ± 10.6 per individual. The average distance between two positions was 37 m (max = 480 m). Toads changed their position on average every 4.1 days. Of all relocations, 95% were within a distance of 756 m to the pond (max = 1,235 m). We did not find an influence of the sex or the body condition (SMI) on d_{total}, d_{mean}, d_{max}, *AI* or mcp_{95} ($p > 0.05$ in all linear models). However, with the exception of d_{mean}, the time the individual was tracked had a significant effect on these parameters ($p < 0.005$ in all linear models).

Four migrations to the forest could be observed during the study time, with arrivals on 12th of May, 3rd of June, 12th of July and 24th of August. Additionally, two of the six toads, which were tracked exclusively in October and November, also reached the forest (24th of October and 16th of November; based on a weekly relocation).

Vineyards as habitat

Of 517 recorded movements 103 (20%) ended in a vineyard (Fig. 2) where toads spent 15% of the total time. In vineyards the mean distance of a hiding place to the vine row was 0.31 m (± 0.32 m) and therefore smaller than expected from a random choice within the vineyard (0.52 ± 0.05 m; Wilcoxon signed-rank test, $W = 1056$, $p < 0.0001$). Toads sitting outside of vineyards could be found in a mean distance of 64 m (± 190 m) to the next vineyard, whereby the distance was > 0 and ≤ 3 m for 7.2% of all relocations. On a new position toads were covered on average by 90% (± 22%), e.g. with vegetation or soil. In 367 and 10 out of 485 cases where an assessment was possible, we found the toad completely with and without cover, respectively.

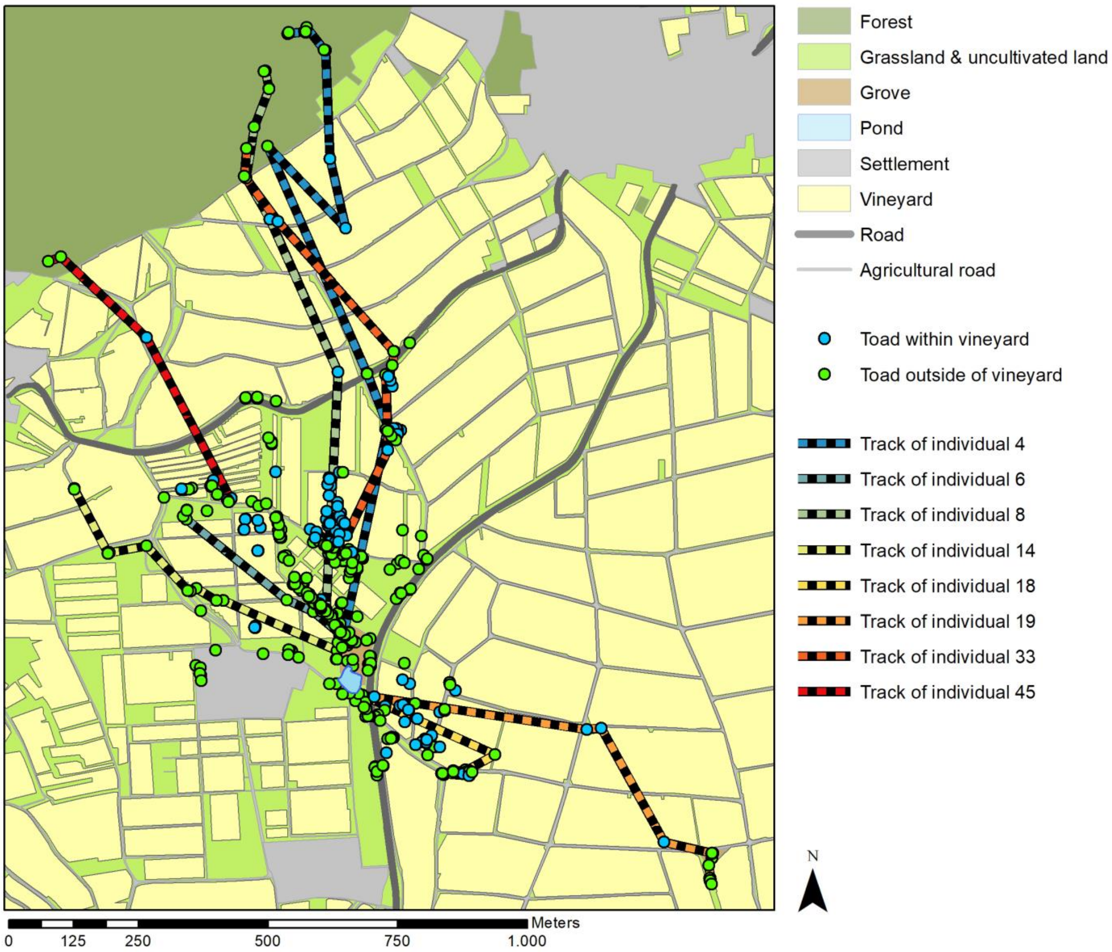


Fig. 2: Positions (n = 516) of all toads that had been tracked between 18th of March and the 7th of September 2017 for at least one day (n = 45). Tracks of different color show examples for the migration paths of eight different individuals, including all individuals that reached the forest (individuals 4, 8, 33 and 45; other shown examples: individuals 6, 14, 18 and 19).

In total, 22 of 51 toads were detected in a vineyard at least once. However, the probability that a toad used a vineyard as habitat during the study period is a function of tracking time (GLM, $p = 0.0014$; Table B and Fig. C in Appendix A). Accordingly, the probability that a toad used a vineyard at least once is 14% after the first day, 54% after the 50th day and 90% after 100 days of tracking. On average 17% (± 14%) of all tracked toads were located within a vineyard per day, but on some days up to two thirds could be found there (Fig. 3A and Appendix C). The time of the year had a significant positive effect on the probability that a toad was detected in a vineyard (GLMM, $p = 0.0051$; Fig. 3B). However, only six individuals were tracked longer than 86 days (mean = 123 ± 29 days) and were therefore considered in this GLMM.

To determine if toads avoid vineyards as habitat, we considered 188 movements, and therefore habitat choices, of 36 individuals. Vineyards were used 23% less often than expected from a random habitat choice (observed: 64; expected: 83), resulting in a significant avoidance of vineyards by toads (Wilcoxon signed-rank test, $W = 113$, $p = 0.0003$). Weather conditions, the handling and a possible disturbance of the toad on the previous day had a significant effect on the probability of a movement of a toad (GLMM, $p < 0.0001$ for all variables), but also the factor vineyard influenced this probability (GLMM, $p = 0.03007$; Table A in Appendix A). When holding the other predictors constant on their mean value, the probability of a movement was 31% higher for toads found in a vineyard. A toad´s disturbance (e.g. tractor) on the previous day increased the probability that a toad changed its position by 224% and a handling (e.g. to check the transmitter) by 196%.

Pesticide applications and spatial-temporal overlap

For the period from the 29th of March (first herbicide applications) to the 30th of August (last fungicide application; 154 days) each of the ten wine growers reported between eight and 12 pesticide applications (mean = 10; see Table C in Appendix A). At least 35 different pesticide formulations with 32 different active ingredients (Table D and E in Appendix A) had been applied, whereby 88% of the used formulations are among the fungicides. Applications took place on 72 different days. Assuming that the pesticide data are representative for the whole study area, seven tracked toads had a probability of over 75% to have been present within a vineyard during a pesticide application at least once (Table 1 and Fig. 4). Taking spray drift or the DT_{50} into account this number increased to nine and to 14 individuals, respectively, and to 15 when both spray drift and the DT_{50} were considered. In the latter scenario, individuals might have been exposed to pesticides or contaminated soil over up to 21 days (Table 1, Fig. 4 and Appendix B). Based on our estimations, up to 9% or 24% (without and with spray drift and DT_{50}) of the reproductive common toad population were exposed to pesticides on a single day (Fig. 3C and Appendix C). Exposures can thus be expected on 61 and 125 different days, respectively.

Table 1: Exposure risk for different scenarios. Mean, SD, min, max and median value for the probability that a toad was exposed to pesticides at least once (exp_p) and the number of coincidences that can be expected (exp_n) for four different scenarios (with and without considering spray drift and the DT_{50} of applied pesticides). See also Fig. 4.

Scenario	exp_p					exp_n				
	mean	**SD**	**min**	**max**	**median**	**mean**	**SD**	**min**	**max**	**median**
Application	0.20	0.35	0.00	0.94	0.00	0.40	0.76	0.00	2.52	0.00
Application + spray drift	0.23	0.36	0.00	0.94	0.00	0.46	0.81	0.00	2.52	0.00
Application + DT_{50}	0.34	0.43	0.00	1.00	0.00	2.89	5.53	0.00	20.70	0.00
Application + spray drift + DT_{50}	0.38	0.44	0.00	1.00	0.10	3.30	5.86	0.00	20.70	0.10

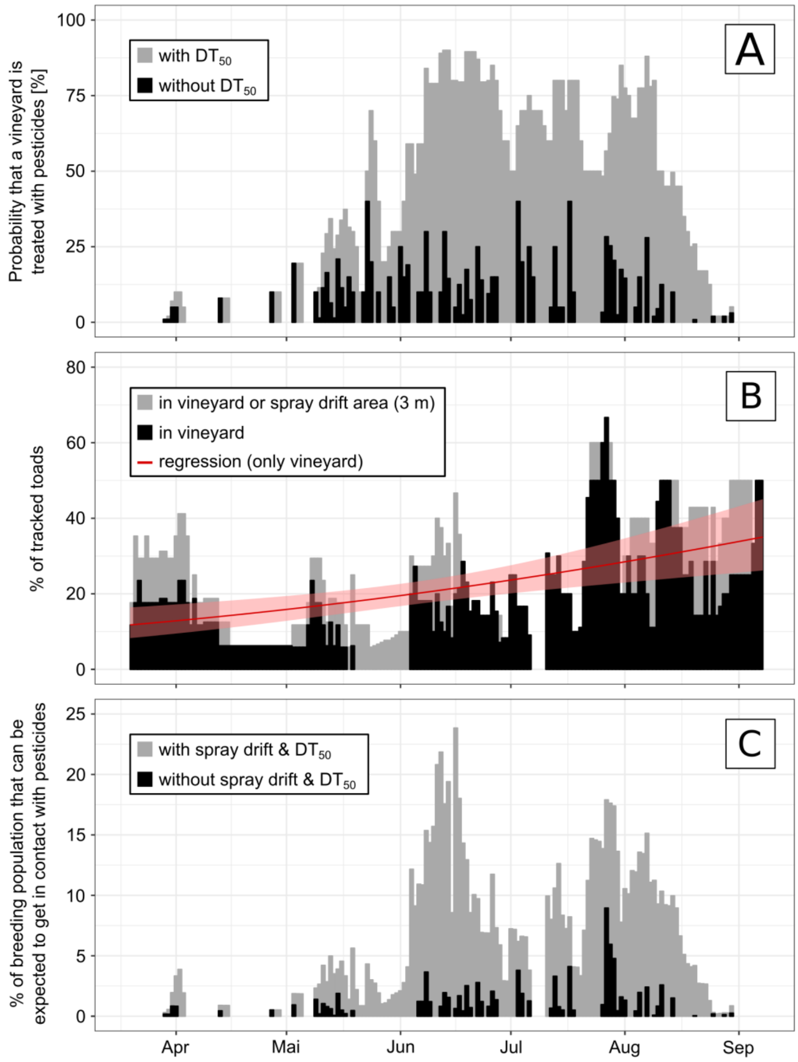


Fig. 3: Pesticide applications, number of individuals in vineyards and exposure risk over the year. (A): Probability that a vineyard was treated or contaminated (considering the DT_{50}) with pesticides for each day of the study. (B): Percentage of the tracked individuals that were found within a vineyard or within a distance of 3 m to the next vineyard for each day. The shown regression is based on six individuals that had been tracked over more than 86 days. (C): Estimated part of the whole breeding population that can be expected to come in contact with pesticides per day. Black bars show the most conservative approach (without spray drift & DT_{50}), whereas the grey bars represent a worst-case-scenario.

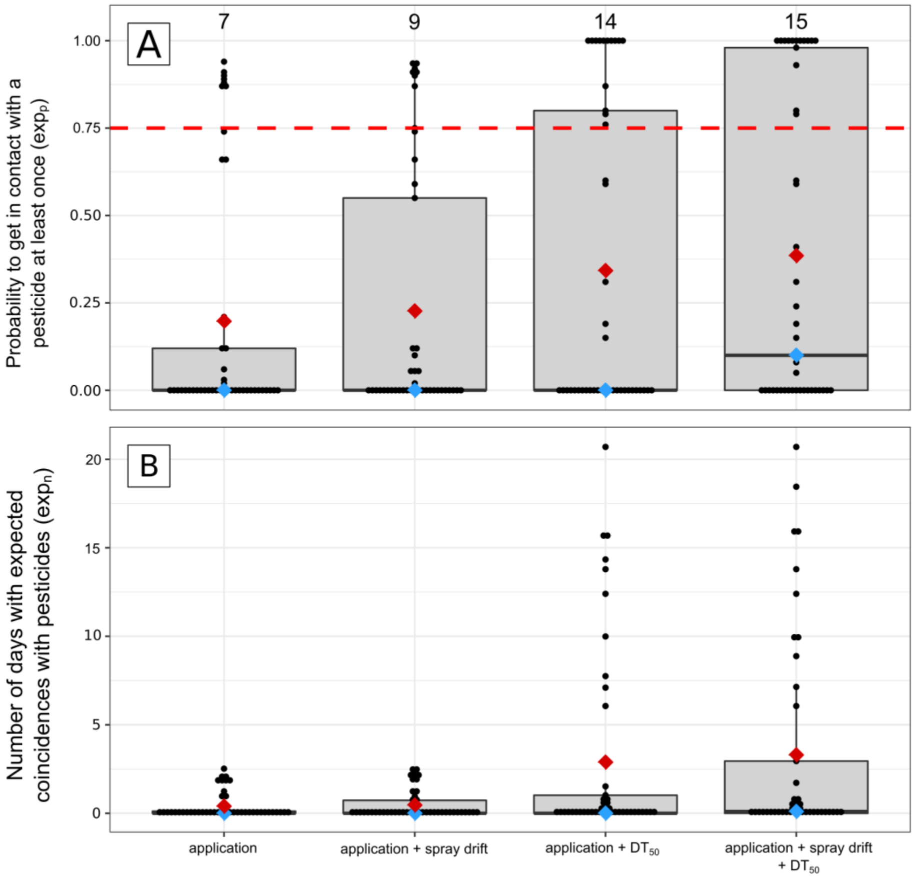


Fig. 4: Boxplots of the exposure risk for different scenarios. The probability that an individual was exposed to pesticides at least once (exp_p; (A)) and the number of coincidences that can be expected (exp_n; (B)) is shown for four different scenarios (with and without considering spray drift and the DT$_{50}$ of applied pesticides). Each black dot represents one individual. Red diamonds show the mean, blue diamonds the median value (see Table 3). Individuals above the red dotted line in (A) have a high probability to be exposed to pesticides (> 75%; number of individuals are shown above the boxplots).

Discussion

By applying telemetry we got an insight into the post-breeding migration of common toads in an agricultural landscape dominated by viticulture. We could show that toads are using the vineyards as temporary habitats during post-breeding migration. Further, we could demonstrate that parts of the population were likely exposed to pesticides during this migration.

Post-breeding migration behavior

Toads reached the Palatinate Forest in May, June, July, August, October and November 2017. These very different arrival times show a high plasticity in the post-breeding migration. Overall, the observed post-breeding migration is in line with previous studies of adult *B. bufo*. In our study 95% of all relocations were within a distance of 756 m to the pond and the maximum distance was 1,235 m. Kovar et al. (2009) found toads in a distance of up to 1,835 m from a breeding pond in regularly mowed meadows in the Czech Republic. Sinsch (1988) described summer habitats in a distances between 55 and 1,600 m in an area covered with pine forest and pastures in West Germany, and Sztatecsny and Schabetsberger (2005) followed toads to a distance of up to 1,000 m to the pond in the Austrian Alps. Daversa et al. (2012) observed a mean distance between two locations of 25 m and a maximum distance of 387 m in a protected mountain region in Spain, which is only a little shorter compared with our results (mean = 37 m, max = 480 m). Also, the detected similar migration behavior of both sexes correspond to existing knowledge (Daversa et al., 2012; Kovar et al., 2009). However, like in many studies on amphibian migration our data are probably underestimating the real movement distance (Sinsch, 2014). For example, we calculated distances between two observations as straight line, ignoring the actual movement path. Moreover, we did not record nocturnal movements, when the individual returns to the same hiding place, and we removed the transmitters of individuals in the Palatinate Forest before the actual hibernacula was reached. However, overall movement distances and home ranges in our agricultural landscape seem to be comparable with those in more natural habitat types.

Vineyards as habitat

Our telemetry data suggests that toads used the vineyards as temporary habitat and can be expected to be within a vineyard at least once during their post-breeding migration. Nevertheless, common toads are not using vineyards as often as expected from random habitat choice and therefore tend to avoid them. By buffering the vineyard-polygons by minus two meters for the calculation, our findings are based on a conservative approach and the actual avoidance might be even more pronounced. An avoidance of arable fields by common toads was also documented by Salazar et al. (2016) in Garford, South East England, where out of 90 individuals that had been found around a pond in a farmland area, not a single one was sitting directly within a cultivated field. Also Vos et al. (2007) showed an avoidance of agricultural areas, as four times more adults and ten times more juveniles were caught in meadows than in arable land when using pitfall traps to study the small scale post-breeding behavior of the common frog (*R. temporaria*) in the Netherlands. In a telemetry study in Spain on natterjack toads (*B. calamita*) agricultural areas were the least preferred habitat type (Miaud and Sanuy, 2005). As a general rule agricultures often have a negative effect on amphibian occurrence and distribution on larger scales (Beja and Alcazar, 2003; Boissinot et al., 2019; Piha et al., 2007; Ray et al., 2002; Suárez et al., 2016), although this depends on the species in focus (Koumaris and Fahrig, 2016; Trochet et al., 2016). For the common toad, Guillot et al. (2016) showed that individuals inhabiting farmlands have a higher body asymmetry than toads from forests, probably because of environmental stress during early life stages.

In our study we observed that the probability of detecting a toad in a vineyard is higher later in the year (Table 3B). At the same time the quality of vineyards as habitat might increase. For example, there is hardly any shade in spring, what changes during the year when grapevine leaves are growing, resulting in a more favorable microclimate for toads. We assume that vineyards are in general suboptimal habitats for toads, especially in spring, and therefore are less often used as habitat. The detected avoidance behavior solely being explained due to chemical contamination is unlikely, as the number of pesticide applications is higher during summer than in the beginning of the migration season (Table 3C). Also laboratory studies demonstrated that terrestrial amphibians are unable to detect contaminants on natural soils (Hatch et al., 2001; Storrs Méndez et al., 2009). However, these studies did not take accumulations of pesticides and multiple applications into account, and were also limited in the exposure period.

The pesticide application process itself (e.g. tractor passes) might also result in regular disturbances of toads sitting in vineyards. Interviewed wine growers reported up to 12 pesticide applications within 116 days, which means one application, i.e. possible disturbance, every 10 days (Table C in Appendix A). Next to pesticide applications also agricultural practices like tillage or mowing can be observed, that might result in an additional disturbance of the toad and even lead to mortality of individuals. In fact, three of the studied toads died because of agricultural processes, two of them directly in vineyards. On the other hand, we observed one toad in a meadow while it was mowed and another one was buried in a vineyard while the soil was ploughed, and both toads survived without obvious injuries. Also Pfeffer et al. (2011) showed that amphibians can survive when they are in a field during a mechanical soil tillage. Agricultural practices might not only be part of the explanation of the general avoidance behavior, but observed disturbances also had a significant effect on the probability of a movement of *B. bufo* (Table A in Appendix A). However, even when we included known disturbances and other factors as exploratory variables in our analysis, the effect of vineyards as habitat on the movement behavior was still significant. This implies that a toad is moving less in other habitats than in vineyards. This might again be an indication of an unsuitable (micro)habitat and/or the effect of pesticides and mechanical practices in the vineyard.

As vineyards are the most dominant agricultural type in the area around our study site (Lenhardt et al., 2013; Statistisches Landesamt Rheinland-Pfalz, 2018; Fig. 1) an avoidance would result in a strong fragmentation of the breeding populations of common toads. Using landscape permeability models Lenhardt et al. (2013) showed that vineyards in our study area indeed lead to a high degree of fragmentation and even the complete isolation of some amphibian populations. However, due to its high migration capacity and the high number of breeding populations in the region, the common toad was comparable little affected from fragmentation than other species in this model. A limited migration would result in decreased gene flow between populations, decreasing the probability of the long-term survival of a population (Allentoft and O'Brien, 2010). Using *R. temporaria* as study species, Lenhardt et al. (2017) showed that vineyards increase the genetic isolation of populations in the region around our study site, and that this isolation results in a decreased genetic diversity. Also Jean-Marc et al. (2018) demonstrated that agriculture can act as a barrier on a larger scale and they concluded that dispersal corridors like rivers, hedgerows and forests are important for survival and dispersal of marbled newts (*Triturus marmoratus*). Therefore, management measures of amphibian populations should not stop at the creation of ponds or the improvement of wetlands, but give also attention to the terrestrial habitat surrounding the breeding site. The importance of the landscape composition and corridors connecting habitats had been discussed in several amphibian studies (e.g. Boissinot et al. (2019), Hartel et al. (2009), Salazar et al. (2016), Sawatzky et al. (2019) or Vos et al., 2007)). For common toads, the characteristic of the terrestrial habitat might even play a more important role than that of the pond (Hartel et al., 2008).

Pesticide applications and spatial-temporal overlap

During common pesticide applications less than 0.1% of the pesticide amount reaches their target organism (Pimentel, 1995) and as a result most agricultural topsoil is contaminated with pesticides (Hvězdová et al., 2018; Silva et al., 2019), even if samples were taken several month after the last application (Hvězdová et al., 2018). To our knowledge, current data on pesticides in Central European vineyards do not exist, but it can be assumed that also in this type of agriculture a large part of the applied pesticides are not reaching their target and thus drift into the environment. Hiding places of toads in vineyards might be particularly contaminated, as we showed that toads are often sitting near the vine row where pesticides are washed down from leaves right above them. Moreover, herbicides are usually applied in an area of up to 30 cm to the vine row (Roßberg and Ipach, 2015). In amphibians, pesticide uptake is highest when individuals are directly oversprayed with pesticides (Van Meter et al., 2015). Direct overspray is a realistic scenario, as we detected one toad in a vineyard during day time outside its hiding place on a day were also pesticide applications were reported (Fig. A in Appendix A). Additionally, we observed several pesticide applications after sunset. As toads are usually night-active (i.e. outside of their hiding places), such evening applications make direct overspray events even more likely. However, an uptake is also possible when amphibians are in contact with contaminated soil (Storrs Méndez et al., 2009; Van Meter et al., 2015). Therefore, toads might be exposed to pesticides even if there is an interception by the grapevines and despite the fact that toads are usually covered.

Although toads prefer other habitats than vineyards, we showed that up to 24% of the breeding population of a pond situated in viticulture is getting in contact with pesticides during a single day. Some individuals are potentially exposed over up to 21 days when considering the DT_{50} and spray drift. Taking spray drift into account might be a worst-case scenario. But even with the spray drift intersection of a vineyard row, pesticide rates over 10% of the field rate are possible within 3 m (Otto et al., 2015). This is a rate that can lead to acute mortality in amphibians for some fungicides in laboratory exposure experiments (Brühl et al., 2013). A high coincidence with pesticide applications during the migration of amphibians using ponds in agricultural areas was also observed in previous studies. Berger et al. (2013) calculated that up to 100% of a population is coming in contact with the herbicide Glyphosate when studying the migration of four temperate amphibian species. Even during the pre-spawning migration up the 86% were exposed when considering all pesticides (Lenhardt et al., 2014). In general, our estimations are following the conservative assumption that all individuals are migrating to areas where they cannot come in contact with pesticides. We also assumed that this migration is a linear function over time. However, as two of six individuals that were tracked in October/November reached the forest, our data suggests that such a migration is for many individuals occurring late in the season, after the last pesticide application in the end of August. This would result in an underestimation of the part of the population that is left in the agricultural landscape and therefore, the percentage of individuals that come in contact with pesticides might be even higher than we calculated. Also locating the toads only during the day might underestimate the exposure risk as toads might be found more often in vineyards during the night, for example when they cross a vineyard to migrate between two hiding places. Therefore we expect that the exposure of a large part of the population is likely, even if our estimations are only based on data of a few individuals.

Several studies demonstrated that pesticides can lead to mortality in terrestrial amphibians when they are directly oversprayed in the laboratory (Belden et al., 2010; Brühl et al., 2013; Relyea, 2005). Also Cusaac et al. (2016) found mortality after a overspray of Blanchard's cricket frogs (*Acris blanchardi*) in the laboratory with the fungicide Headline AMP at concentrations similar to the suggested label rate. However, no increased mortality was observed in a semi field study with the same fungicide, concentration and study species, but fungicides were applied aerially with planes and concentration at ground level was ≤ 19% of the field rate (Cusaac et al., 2015). Cusaac et al. (2017) could show that soil previously exposed to Headline AMP can also lead to mortality in the toad *Anaxyrus cognatus*. The median lethal dose was 64% higher than the maximum recommended label rate (Cusaac et al., 2017), but this might be a realistic worst-case scenario on arable fields. In the present study, we could not observe direct mortality that can be traced back to a pesticide application. However, juveniles, which are usually tested in laboratory studies, might in general be more sensitive to pesticides than adults (Cusaac et al., 2017). This is in line with Swanson et al. (2018), where a comparable high degree of mortality was observed during the tracking of northern leopard frogs (*Lithobates pipiens*), but all of them could be linked to predation events. However, although *L. pipiens* spent just 6% of their time in agricultural fields, pesticide accumulations were found when analyzing frog tissue (Swanson et al., 2018). Such uptakes might result in sublethal effects, like altered biochemical processes in the liver (Van Meter et al., 2018), a decreased brain cholinesterase activity (Henson-Ramsey et al., 2008), alterations in the hepatic tissue and erythrocyte nuclear abnormalities (Franco-Belussi et al., 2016) or an increased fatigue (Mitchkash et al., 2014). Studies on uptakes and effects of pesticides used in vineyards on terrestrial amphibians are scarce. However, several of these pesticides are generally considered as potentially harmful. For example folpet, the most widely used fungicide, is considered as skin-irritating (see Table D in Appendix A). Therefore, the presence of *B. bufo* during or shortly after a pesticide application might also lead to pesticide accumulations and sublethal effects, even if toads are spending only some days within vineyards.

The general trend in viticulture is moving in the direction of organic farming (Provost and Pedneault, 2016; Willer and Lernoud, 2008). However, also in this management type plant protection products are used. Unfortunately, also the actual effects of most organic plant protection products on terrestrial amphibians remain unclear, as data on toxicity are lacking. In organic farming, copper is often used as replacement for conventional fungicides (Gessler et al., 2011), resulting in a two times higher copper concentration in the soil compared to conventional vineyards in Southern Palatinate (Steinmetz et al., 2017). It is known that copper has negative effects on amphibians in the aquatic stage (García-Muñoz et al., 2009; Wang et al., 2016). Bazar et al. (2009) showed that high copper concentrations in the soil can have lethal effects on terrestrial red-backed salamanders (*Plethodon cinereus*). Tested concentrations were below concentrations documented by Steinmetz et al. (2017) for soils in vineyards, but here the majority of copper was bound to soil organic matter. However, direct overspray or the contact with recently oversprayed soil might harm amphibians. Therefore, future studies should also focus on the effects that plant protection products used in organic farming can have on terrestrial amphibians. While the trend to organic farming can be generally considered as advantageous (Hole et al., 2005; Puig-Montserrat et al., 2017), the danger for amphibians might not decrease at the same scale. However, a diverse landscape with uncultivated patches and suitable corridors might still be more important than the management type of vineyards.

Conclusion

Common toads tend to avoid vineyards, but they are still using them occasionally as habitat. Therefore they might be exposed to pesticides. This combination - exposure despite avoidance -

might have possible negative effects from the individual (i.e. sublethal to lethal effects due to contact with pesticides) to the (meta)population level (i.e. restricted migration and therefore reduced gene flow between populations). There is a knowledge gap about the toxicity of most conventional, but also of organic plant protection products on terrestrial life phases of amphibians. Following the precautionary principle we generally recommend the reduction of pesticide applications to decrease the exposure risk of amphibians using vineyards as habitat. Reductions only during certain time windows intending to allow toads a safe migration, might not be effective because of the high plasticity in the post-breeding migration. However, it might be worth to reduce applications when juveniles emerge out of the water or when amphibians are generally more active (e.g. during or shortly after rain events or after sunset). A heterogeneous cultural landscape with buffer strips around ponds, uncultivated patches and migration corridors that connect ponds and suitable terrestrial habitats might be the best management measure. Finally, we encourage researchers to perform similar studies with different amphibian species, in different types of agriculture and in different regions, to provide more basic data which can be used for developing a general pesticide risk assessment for amphibians.

Additional Information and Declarations

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Author Contributions

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Carsten Brühl: Conceptualization, Supervision, Writing review & editing.

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Supporting information

Appendix A . Additional tables and figures.

Figure A: A common toad (*Bufo bufo*) that was found in a vineyard outside its hiding place in the afternoon of the 27th of July 2017. Four of ten wine growers reported pesticide applications on this day (see Table C), making a direct overspraying of toads likely. The telemetry transmitter and parts of the mounting belt can be seen.

Table A: Detailed result of the GLMM relating the probability of a toad's movement and variables describing the weather, a disturbance and a handling the day before and if the toad was sitting in a vineyard or not.

Coefficient	Estimate	Std. Error	z value	Pr(>|z|)	
(Intercept)	-1.241	0.1405	-8.836	< 0.0001	***
Sum precipitation [1]	0.246	0.0615	3.999	< 0.0001	***
Mean humidity [1]	0.244	0.061	3.996	< 0.0001	***
Min Temperature [1]	0.577	0.082	7.002	< 0.0001	***
Min Temperature^2 [1]	-0.235	0.073	-3.209	0.00133	**
Disturbance [2]	2.504	0.413	6.055	< 0.0001	***
Handling [2]	2.076	0.286	7.260	< 0.0001	***
Vineyard	0.375	0.173	2.169	0.03007	*

[1] During the night/day before the potential movement (24 h, starting at 6 am on the day before the relocation). Data with a temporal resolution of 1 h were received from the weather station "Siebeldingen 88 / SBD".

[2] On the day before the potential movement

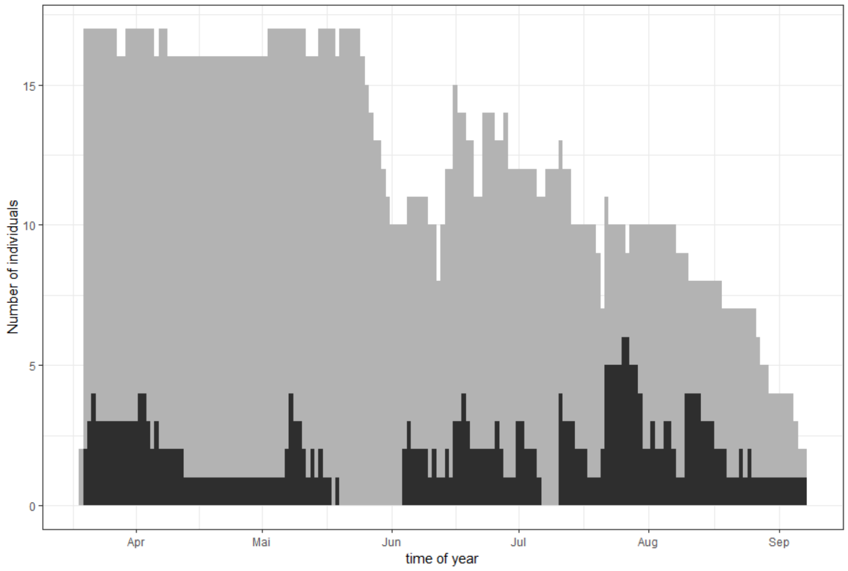

Figure B: Number of individuals that had been tracked for each day between the 18th of March and 7th of September 2017 (bright bars) and number of individuals that had been found in a vineyard (dark bars). Altogether 51 toads had been tracked.

Table B: Detailed result of the GLM relating if a toad used a vineyard as habitat as least once with the days the individuals had been tracked.

Coefficients	Estimate	Std. Error	z value	Pr(>|z|) value	
(Intercept)	-1.817	0.630	-2.886	0.0039	**
Days tracked	0.040	0.012	3.199	0.0014	**

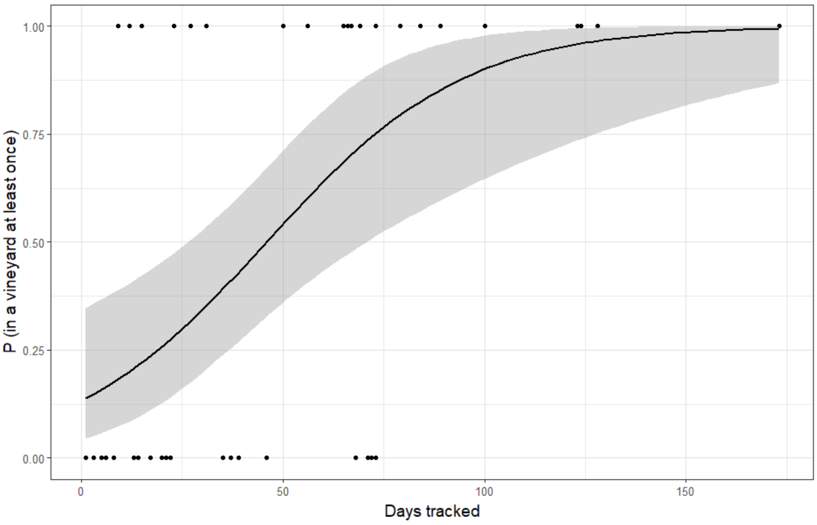

Figure C: The probability to be found in a vineyard at least once as a function of days an individual had been tracked (grey area = 95% CI). Dots are representing individuals that had been tracked for at least one day (n = 45).

Table C: Pesticide applications that were reported from local wine growers (n = 10). The assumed persistence is based on the DT_{50} of the active indigent of the formulation (see Table D). When a wine grower reported that an application of all vineyards was spread over two or more days, each day was weighted accordingly ("Part"). For formulations in quotation marks the exact name of the formulation was not clear. Fertilisers and wetting agents were not taken into account.

Wine grower	Date	Applied pesticides	Part	Highest DT_{50}	Assumed persistence
A	29.03.2017	Durano TF (Glyphosate)	0.10	23.79	3
A	30.03.2017	Durano TF (Glyphosate)	0.10	23.79	3
A	31.03.2017	THIOVIT JET (Sulfur)	0.50	3	3
A	01.04.2017	THIOVIT JET (Sulfur)	0.50	3	3
A	10.05.2017	THIOVIT JET (Sulfur), Polyram® WG (Metiram)	0.14	7	3
A	11.05.2017	THIOVIT JET (Sulfur), Polyram® WG (Metiram)	0.14	7	3
A	12.05.2017	THIOVIT JET (Sulfur), Polyram® WG (Metiram)	0.14	7	3
A	13.05.2017	THIOVIT JET (Sulfur), Polyram® WG (Metiram)	0.14	7	3
A	14.05.2017	THIOVIT JET (Sulfur), Polyram® WG (Metiram)	0.14	7	3
A	15.05.2017	THIOVIT JET (Sulfur), Polyram® WG (Metiram)	0.14	7	3
A	16.05.2017	THIOVIT JET (Sulfur), Polyram® WG (Metiram)	0.14	7	3
A	23.05.2017	Kumulus® WG (Sulfur), Polyram® WG (Metiram)	1.00	7	3
A	01.06.2017	Vivando® (Metrafenone), Folpan® 80 WDG (Folpet)	0.50	146	14
A	02.06.2017	Vivando® (Metrafenone), Folpan® 80 WDG (Folpet)	0.50	146	14
A	08.06.2017	Luna® Experience (Fluopyram, Tebuconazole), RIDOMIL GOLD® MZ (Metalaxyl-M, Mancozeb)	0.50	118.8	14
A	09.06.2017	Luna® Experience (Fluopyram, Tebuconazole), RIDOMIL GOLD® MZ (Metalaxyl-M, Mancozeb)	0.50	118.8	14
A	17.06.2017	Talendo® (Proquinazid), Orvego® (Ametoctradin, Dimethomorph)	0.25	44	3
A	18.06.2017	Talendo® (Proquinazid), Orvego® (Ametoctradin, Dimethomorph)	0.25	44	3
A	19.06.2017	Talendo® (Proquinazid), Orvego® (Ametoctradin, Dimethomorph)	0.25	44	3
A	20.06.2017	Talendo® (Proquinazid), Orvego® (Ametoctradin, Dimethomorph)	0.25	44	3
A	26.06.2017	STEWARD (Indoxacarb), Collis® (Boscalid , Kresoximmethyl), Electis MZ (Zoxamide , Mancozeb)	0.10	118	14
A	26.06.2017	Collis® (Boscalid , Kresoximmethyl), Electis MZ (Zoxamide , Mancozeb)	0.40	118	14
A	27.06.2017	STEWARD (Indoxacarb), Collis® (Boscalid , Kresoximmethyl), Electis MZ (Zoxamide , Mancozeb)	0.10	118	14
A	27.06.2017	Collis® (Boscalid , Kresoximmethyl), Electis MZ (Zoxamide , Mancozeb)	0.40	118	14
A	06.07.2017	Vegas (Cyflufenamid), Vinostar® (Folpet, Dimethomorph)	0.50	44	3
A	07.07.2017	Vegas (Cyflufenamid), Vinostar® (Folpet, Dimethomorph)	0.50	44	3
A	17.07.2017	Kusabi (Pyriofenone), Fantic® F (Folpet, Benalaxyl-M)	1.00	44	3
A	28.07.2017	Talendo® (Proquinazid), Folpan® 80 WDG (Folpet)	0.25	30.5	3
A	29.07.2017	Talendo® (Proquinazid), Folpan® 80 WDG (Folpet)	0.25	30.5	3
A	30.07.2017	Talendo® (Proquinazid), Folpan® 80 WDG (Folpet)	0.25	30.5	3
A	31.07.2017	Talendo® (Proquinazid), Folpan® 80 WDG (Folpet)	0.25	30.5	3
A	09.08.2017	Systhane 20 EW (Myclobutanil), Folpan® 80 WDG (Folpet), Kumar (Potassium bicarbonate), Funguran progress (Copper hydroxid)	0.20	35	3
A	28.08.2017	"Spintor" (Spinosad)	0.20	3.3	3
B	12.05.2017	Polyram® WG (Metiram), "Sulfur" (Sulfur)	0.50	7	3
B	13.05.2017	Polyram® WG (Metiram), "Sulfur" (Sulfur)	0.50	7	3
B	19.05.2017	"Roundup" (Glyphosate)	1.00	23.79	3
B	23.05.2017	Polyram® WG (Metiram), "Sulfur" (Sulfur)	1.00	7	3
B	06.06.2017	"Dithane" (Mancozeb), "Sulfur" (Sulfur)	1.00	3	3
B	19.06.2017	"Dithane" (Mancozeb), "Sulfur" (Sulfur)	1.00	3	3
B	03.07.2017	Fantic® F (Folpet, Benalaxyl-M), Collis® (Boscalid , Kresoximmethyl)	0.50	118	14
B	04.07.2017	Fantic® F (Folpet, Benalaxyl-M), Collis® (Boscalid , Kresoximmethyl)	0.50	118	14
B	14.07.2017	Fantic® F (Folpet, Benalaxyl-M), Luna® Experience (Fluopyram, Tebuconazole)	0.50	118.8	14
B	15.07.2017	Fantic® F (Folpet, Benalaxyl-M), Luna® Experience (Fluopyram, Tebuconazole)	0.50	118.8	14
B	27.07.2017	"Folpan" (Folpet), Collis® (Boscalid , Kresoximmethyl)	0.50	118	14
B	28.07.2017	"Folpan" (Folpet), Collis® (Boscalid , Kresoximmethyl)	0.50	118	14
B	01.08.2017	"Roundup" (Glyphosate)	1.00	23.79	3
B	04.08.2017	"Folpan" (Folpet), Topas® (Penconazole)	0.50	90	14
B	05.08.2017	"Folpan" (Folpet), Topas® (Penconazole)	0.50	90	14
C	11.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
C	18.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
C	29.05.2017	"Netzschwefel" (Sulfur), "Dithane" (Mancozeb), "Phosphorous acid" (Phosphorous acid)	1.00	3	3
C	08.06.2017	DYNALI® (Cyflufenamid, Difenoconazol), RIDOMIL GOLD® MZ (Metalaxyl-M, Mancozeb)	1.00	85	14
C	14.06.2017	Luna® Experience (Fluopyram, Tebuconazole), Mildicut® (Cyazofamid)	0.50	118.8	14
C	16.06.2017	Luna® Experience (Fluopyram, Tebuconazole), Mildicut® (Cyazofamid)	0.50	118.8	14
C	22.06.2017	Vivando® (Metrafenone), "Folpan" (Folpet)	0.50	146	14
C	23.06.2017	Vivando® (Metrafenone), "Folpan" (Folpet)	0.50	146	14
C	03.07.2017	Talendo® (Proquinazid), "Folpan" (Folpet)	1.00	30.5	3
C	13.07.2017	Kusabi (Pyriofenone), "Folpan" (Folpet)	1.00	19	3
C	27.07.2017	VENTO™ Power (Myclobutanil, Quinoxyfen), "Folpan" (Folpet)	1.00	169.3	14
C	07.08.2017	"Folpan" (Folpet), Systhane 20 EW (Myclobutanil)	1.00	35	3
D	17.05.2017	Polyram® WG (Metiram), "Netzschwefel" (Sulfur)	0.50	7	3
D	18.05.2017	Polyram® WG (Metiram), "Netzschwefel" (Sulfur)	0.50	7	3
D	29.05.2017	Polyram® WG (Metiram), "Netzschwefel" (Sulfur)	0.50	7	3
D	30.05.2017	Polyram® WG (Metiram), "Netzschwefel" (Sulfur)	0.50	7	3

Wine grower	Date	Applied pesticides	Part	Highest DT_{50}	Assumed persistence
D	08.06.2017	Enervin® (Ametoctradin, Metiram), Vivando® (Metrafenone)	0.50	146	14
D	09.06.2017	Enervin® (Ametoctradin, Metiram), Vivando® (Metrafenone)	0.50	146	14
D	19.06.2017	Mildicut® (Cyazofamid) , Collis® (Boscalid , Kresoximmethyl)	0.50	118	14
D	20.06.2017	Mildicut® (Cyazofamid), Collis® (Boscalid , Kresoximmethyl)	0.50	118	14
D	03.07.2017	FORUM®GOLD (Dithianon, Dimethomorph), Vivando® (Metrafenone)	0.50	146	14
D	04.07.2017	FORUM®GOLD (Dithianon, Dimethomorph), Vivando® (Metrafenone)	0.50	146	14
D	12.07.2017	"Folpan" (Folpet), DYNALI® (Cyflufenamid, Difenoconazol)	0.50	85	14
D	13.07.2017	"Folpan" (Folpet), DYNALI® (Cyflufenamid, Difenoconazol)	0.50	85	14
D	26.07.2017	"Folpan" (Folpet), Systhane 20 EW (Myclobutanil)	0.33	35	3
D	27.07.2017	"Folpan" (Folpet), Systhane 20 EW (Myclobutanil)	0.33	35	3
D	28.07.2017	"Folpan" (Folpet), Systhane 20 EW (Myclobutanil)	0.33	35	3
D	07.08.2017	"Folpan" (Folpet), Systhane 20 EW (Myclobutanil)	1.00	35	3
E	13.04.2017	"Glyphosat" (Glyphosate)	0.80	23.79	3
E	03.05.2017	"Netzschwefel" (Sulfur), "Tridex" (Mancozeb)	0.95	3	3
E	15.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
E	24.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
E	01.06.2017	DYNALI® (Cyflufenamid, Difenoconazol), Polyram® WG (Metiram)	1.00	85	14
E	07.06.2017	Vivando® (Metrafenone), Profiler® (Fluopicolide , Fosetyl-aluminium)	1.00	146	14
E	13.06.2017	Luna® Experience (Fluopyram, Tebuconazole), Orvego® (Ametoctradin, Dimethomorph)	1.00	118.8	14
E	26.06.2017	Talendo® (Proquinazid), "Folpan" (Folpet)	1.00	30.5	3
E	07.07.2017	Vivando® (Metrafenone), "Folpan" (Folpet)	1.00	146	14
E	17.07.2017	Collis® (Boscalid , Kresoximmethyl), "Folpan" (Folpet)	1.00	118	14
E	28.07.2017	Topas® (Penconazole), "Folpan" (Folpet)	0.95	90	14
E	07.08.2017	Topas® (Penconazole), "Folpan" (Folpet)	0.80	90	14
F	16.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
F	24.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram), Talendo® (Proquinazid)	1.00	30.5	3
F	03.06.2017	DYNALI® (Cyflufenamid, Difenoconazol), Polyram® WG (Metiram)	0.90	85	14
F	13.06.2017	Luna® Experience (Fluopyram, Tebuconazole), Fantic® F (Folpet, Benalaxyl-M)	1.00	118.8	14
F	23.06.2017	Kusabi (Pyriofenone), "Folpan" (Folpet)	0.90	19	3
F	03.07.2017	Collis® (Boscalid , Kresoximmethyl), "Folpan" (Folpet)	1.00	118	14
F	17.07.2017	Kusabi (Pyriofenone)	1.00	19	3
F	31.07.2017	Topas® (Penconazole)	1.00	90	14
F	01.08.2017	Topas® (Penconazole)	0.46	90	14
F	20.08.2017	TELDOR (Fenhexamid)	0.09	0.43	1
F	30.08.2017	TELDOR (Fenhexamid)	0.31	0.43	1
G	12.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
G	22.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
G	02.06.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
G	12.06.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
G	22.06.2017	Folpan® 80 WDG (Folpet), VENTO™ Power (Myclobutanil, Quinoxyfen), "Netzschwefel" (Sulfur)	1.00	169.3	14
G	04.07.2017	Folpan® 80 WDG (Folpet), Talendo® (Proquinazid), "Netzschwefel" (Sulfur)	1.00	30.5	3
G	29.07.2017	Folpan® 80 WDG (Folpet), Talendo® (Proquinazid)	0.50	30.5	3
G	31.07.2017	Folpan® 80 WDG (Folpet), Talendo® (Proquinazid)	0.50	30.5	3
G	05.08.2017	Durano TF (Glyphosate)	1.00	23.79	3
G	14.08.2017	Folpan® 80 WDG (Folpet), Talendo® (Proquinazid)	1.00	30.5	3
H	15.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	0.95	7	3
H	26.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
H	08.06.2017	Vivando® (Metrafenone), Fantic® F (Folpet, Benalaxyl-M)	1.00	146	14
H	17.06.2017	Luna® Experience (Fluopyram, Tebuconazole), RIDOMIL GOLD® MZ (Metalaxyl-M, Mancozeb)	1.00	118.8	14
H	27.06.2017	Talendo® (Proquinazid), "Folpan" (Folpet)	1.00	30.5	3
H	06.07.2017	DYNALI® (Cyflufenamid, Difenoconazol), "Folpan" (Folpet)	1.00	85	14
H	17.07.2017	Collis® (Boscalid , Kresoximmethyl), VitiSan® (Potassium bicarbonate), "Folpan" (Folpet)	1.00	118	14
H	28.07.2017	Vivando® (Metrafenone), "Folpan" (Folpet)	0.50	146	14
H	29.07.2017	Vivando® (Metrafenone), "Folpan" (Folpet)	0.50	146	14
H	10.08.2017	Collis® (Boscalid , Kresoximmethyl), Mildicut® (Cyazofamid)	0.45	118	14
H	11.08.2017	Collis® (Boscalid , Kresoximmethyl), Mildicut® (Cyazofamid)	0.45	118	14
I	03.05.2017	"Netzschwefel" (Sulfur), STEWARD (Indoxacarb), Dithane NeoTec (Mancozeb)	1.00	5.97	3
I	23.05.2017	"Netzschwefel" (Sulfur), Polyram® WG (Metiram)	1.00	7	3
I	03.06.2017	DYNALI® (Cyflufenamid, Difenoconazol), Polyram® WG (Metiram)	1.00	85	14
I	14.06.2017	Vivando® (Metrafenone), Mildicut® (Cyazofamid)	0.95	146	14
I	25.06.2017	Talendo® (Proquinazid), "Folpan" (Folpet)	0.95	30.5	3
I	06.07.2017	VENTO™ Power (Myclobutanil, Quinoxyfen), "Folpan" (Folpet)	1.00	169.3	14
I	18.07.2017	Vegas (Cyflufenamid), "Folpan" (Folpet)	1.00	25.3	3
I	29.07.2017	Collis® (Boscalid , Kresoximmethyl), "Folpan" (Folpet)	0.80	118	14
I	11.08.2017	Vivando® (Metrafenone), Mildicut® (Cyazofamid)	0.80	146	14
I	25.08.2017	"Spintor" (Spinosad)	0.20	3.3	3
J	27.04.2017	"Sulfur" (Sulfur), Delan® WG (Dithianon)	1.00	35	3
J	09.05.2017	"Sulfur" (Sulfur), Dithane NeoTec (Mancozeb)	1.00	3	3
J	23.05.2017	"Netzschwefel" (Sulfur), "Folpan" (Folpet), Topas® (Penconazole)	1.00	90	14
J	01.06.2017	"Folpan" (Folpet), DYNALI® (Cyflufenamid, Difenoconazol)	1.00	85	14
J	13.06.2017	"Folpan" (Folpet), Vivando® (Metrafenone)	1.00	146	14
J	22.06.2017	"Folpan" (Folpet), Luna® Experience (Fluopyram, Tebuconazole)	1.00	118.8	14
J	03.07.2017	"Folpan" (Folpet), Talendo® (Proquinazid)	1.00	30.5	3
J	13.07.2017	"Folpan" (Folpet), DYNALI® (Cyflufenamid, Difenoconazol)	1.00	85	14
J	27.07.2017	"Folpan" (Folpet), Collis® (Boscalid , Kresoximmethyl)	1.00	118	14

Table D: List of active substances that can be found in formulations applied by local wine growers in 2017 (see Table E). Information of each substance was obtained from the Pesticide Properties DataBase (PPDB; https://sitem.herts.ac.uk/aeru/ppdb/en/ - accessed on 24th of February 2019). If available, the half life in soil (DT_{50} soil) from field studies (DT50(field)) was used. If not, the DT50(typical) was used (marked with *). For substances for which there was no entry in the database, a DT_{50} of 3 days was assumed (marked with **).

Active substance	CAS nr.	Type	Substance group	Molecular mass [g mol^{-1}]	log k_{ow}	k_{oc}	k_{foc}	DT_{50} aqueous hydrolysis [days]	DT_{50} soil [days]	Skin irritant
Ametoctradin	865318-97-4	Fungicide	Triazolopyrimidine	275.39	4.4	7713	3779	Stable	19.7	No
Benalaxyl-M	98243-83-5	Fungicide	Phenylamide	325.40	3.68	7175	6063	Stable	44	no
Boscalid	188425-85-6	Fungicide	Carboxamide	343.21	2.96	-	772	Stable	118	no
Copper hydroxid **	20427-59-2	Fungicide	-	-	-	-	-	-	3	-
Cyazofamid	120116-88-3	Fungicide	Cyanoimidazole	324.78	3.2	-	1338	0.1	4.5	no
Cyflufenamid	180409-60-3	Fungicide	Amidoxine	412.36	4.7	-	1592	Stable	25.3	yes
Difenoconazol	119446-68-3	Fungicide	Triazole	406.26	4.36	-	3760	Stable	85	yes
Dimethomorph	110488-70-5	Fungicide	Morpholine	387.86	2.68	-	419.4	70	44	yes
Dithianon	3347-22-6	Fungicide	Quinone	296.32	3.2	3627	-	0.6	35	no
Fenhexamid *	126833-17-8	Fungicide	Hydroxyanilide	302.20	3.51	475	733.5	Stable	0.43	no
Fluopicolide	239110-15-7	Fungicide	Benzamide	383.58	2.9	-	321.1	Stable	138.8	no
Fluopyram	658066-35-4	Fungicide	Benzamide, pyramide	396.76	3.3	-	278.9	Stable	118.8	no
Folpet	133-07-3	Fungicide	Phthalimide	296.56	3.02	304	-	0.05	3	yes
Fosetyl-aluminium *	39148-24-8	Fungicide	Organophosphate	354.10	-2.1	-	-	Stable	0.1	no
Glyphosate	1071-83-6	Herbicide	Phosphonoglycine	169.1	-3.2	1424	16331	Stable	23.79	yes
Indoxacarb	173584-44-6	Insecticide	Oxadiazine	527.83	4.65	4483	-	17.6	5.97	no
Kresoximmethyl *	143390-89-0	Fungicide	Strobilurin	313.35	3.4	-	308	35	16	yes
Mancozeb *	8018-01-7	Fungicide	Carbamate	271.3	2.3	998	771	1.3	0.05	unknown
Metalaxyl-M	70630-17-0	Fungicide	Phenylamide	279.33	1.71	-	78.9	Stable	14.1	yes
Metiram	9006-42-2	Fungicide	Carbamate	1088.6	0.33	903012	-	0.1	7	no
Metrafenone	220899-03-6	Fungicide	Benzophenone	409.27	4.3	7061	3105	Stable	146	yes
Myclobutanil	88671-89-0	Fungicide	Triazole	288.78	2.89	-	517	Stable	35	no
Penconazole	66246-88-6	Fungicide	Triazole	284.18	3.72	-	2205	Stable	90	no
Phosphorous acid **	13598-36-2	Fungicide	-	-	-	-	-	-	3	-.
Potassium bicarbonate **	298-14-6	Fungicide	-	-	-	-	-	-	3	-.
Proquinazid	189278-12-4	Fungicide	Quinazolinone	372.2	5.5	-	12870	Stable	30.5	no
Pyriofenone	688046-61-9	Fungicide	Benzoylpyridine	365.81	3.2	-	1485	Stable	19	no
Quinoxyfen	124495-18-7	Fungicide	Quinoline	308.13	5.1	-	22929	Stable	169.3	unknown
Spinosad	168316-95-8	Insecticide	Micro-organism derived	731.98+745.98	4.1	34600	-	Stable	3.3	no
Sulfur **	7704-34-9	Fungicide	-	-	-	-	-	-	3	-
Tebuconazole	107534-96-3	Fungicide	Triazole	307.82	3.7	-	769	Stable	47.1	no
Zoxamide	156052-68-5	Fungicide	Benzamide	336.64	3.76	1224	1224.2	15.7	6	unknown

Table E: List of formulations that were used by local wine growers (see Table C). Information for each formulation were taken from the user manuals. Recommended field rates are depending on the intended use and the stage of development of the vine. Therefore ranges are given. For formulations in quotation marks the exact name of the formulation was not clear.

Formulation	Type	Active substances	Field rate	Max. applications per year
Collis®	Fungicide	Boscalid , Kresoximmethyl	0.16 - 0.64 l/ha	3
Delan® WG	Fungicide	Dithianon	0.2 - 0.8 kg/ha	8
Dithane NeoTec	Fungicide	Mancozeb	0.8 - 3.2 kg/ha	6
Durano TF	Herbicide	Glyphosate	5 l/ha	2
DYNALI®	Fungicide	Cyflufenamid, Difenoconazol	0.15 - 0.65 l/ha	2
Electis MZ	Fungicide	Mancozeb, Zoxamide	0.72 - 2.88 kg/ha	4
Enervin®	Fungicide	Ametoctradin, Metiram	0.75 - 4 kg/ha	3
Fantic® F	Fungicide	Folpet, Benalaxyl-M	0.45 - 2.4 kg/ha	3
Folpan® 80 WDG	Fungicide	Folpet	0.4 - 1.6 kg/ha	8
FORUM®GOLD	Fungicide	Dithianon, Dimethomorph	0.48 - 1.56 kg/ha	3
Funguran progress	Fungicide	Copper hydroxid	2 kg/ha	4
Kumar	Fungicide	Potassium bicarbonate	1.25 - 5 kg/ha	6
Kumulus® WG	Fungicide	Sulfur	2.4 - 4.8 kg/ha	8
Kusabi	Fungicide	Pyriofenone	0.075 - 0.3 l/ha	3
Luna® Experience	Fungicide	Fluopyram, Tebuconazole	0.09 - 0.4 l/ha	2
Mildicut®	Fungicide	Cyazofamid	0.75 - 4 l/ha	3
Orvego®	Fungicide	Ametoctradin, Dimethomorph	0.4 - 1.6 l/ha	3
Polyram® WG	Fungicide	Metiram	0.8 - 3.2 kg/ha	6
Profiler®	Fungicide	Fluopicolide , Fosetyl-aluminium	1.5 - 3 kg/ha	2
RIDOMIL GOLD® MZ	Fungicide	Metalaxyl-M, Mancozeb	0.54 - 1.44 kg/ha	2
STEWARD	Insecticide	Indoxacarb	0.125 - kg/ha	3
Systhane 20 EW	Fungicide	Myclobutanil	0.12 - 0.24 l/ha	4
Talendo®	Fungicide	Proquinazid	0.1 - 0.375 l/ha	4
TELDOR	Fungicide	Fenhexamid	0.5 - 1.6 kg/ha	2
THIOVIT JET	Fungicide	Sulfur	2 - 8 kg/ha	10
Topas®	Fungicide	Penconazole	0.32 - l/ha	4
Vegas	Fungicide	Cyflufenamid	0.09 - 0.5 l/ha	2
VENTO™ Power	Fungicide	Myclobutanil, Quinoxyfen	0.4 - 1.6 l/ha	4
Vinostar®	Fungicide	Folpet, Dimethomorph	0.5 - 2 kg/ha	3
VitiSan®	Fungicide	Potassium bicarbonate	3 - 12 kg/ha	6
Vivando®	Fungicide	Metrafenone	0.08 - 0.32 l/ha	3
"Dithane"	Fungicide	Mancozeb	Exact formulation not known	
"Folpan"	Fungicide	Folpet		
"Glyphosat"	Herbicide	Glyphosate		
"Netzschwefel"	Fungicide	Sulfur		
"Phosphorous acid"	Fungicide	Phosphorous acid		
"Roundup"	Herbicide	Glyphosate		
"Spintor"	Insecticide	Spinosad		
"Sulfur"	Fungicide	Sulfur		
"Tridex"	Fungicide	Mancozeb		

Appendix B. Detailed information about each individual.

Details about the tracked toads and their post-breeding migration behavior. Reasons for ending the tracking: A = Death caused by agricultural practice; E = End of study period or > 1 month in forest; I = skin injury caused by the transmitter; L = Signal lost; O = Other injury; P = predation; S = the transmitter was shed; T = Death caused by road traffic; U = unknown cause of death.

Indi	Sex	SVL [mm]	Weight [g]	Release date	Last relocation	Number of relocations	Different positions	AI	d_{total} [m]	d_{max} [m]	d_{mean} [m]	mcp_{95} [m²]	d_{pond_max} [m]	D_{pond_mean} [m]	Days in vineyard	exp_p application	exp_n application	exp_p application + spray drift	exp_n application + spray drift	exp_p application + DT_{50}	exp_n application + DT_{50}	exp_p application + spray drift + DT_{50}	exp_n application + spray drift + DT_{50}	Reason ending	Comment
1	W	98	71.1	18.03.2017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	
2	W	90	58	18.03.2017	07.09.2017	173	60	0.35	1,5	137	26	7,594	301	205	27	0.90	2.05	0.91	2.15	1.00	15.69	1.00	15.99	E	
3	W	87	58	18.03.2017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	
4	W	88	61.6	18.03.2017	20.07.2017	124	29	0.23	1,8	480	62	71,66	1,235	710	20	0.87	1.85	0.87	1.85	1.00	12.40	1.00	12.40	E	Forest on02.06.2017
5	W	101	95.4	18.03.2017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	
6	W	80	50.9	19.03.2017	27.03.2017	8	6	0.75	401	248	67	4,079	432	359	0	0	0	0	0	0	0	0	0	S	
7	W	79	42.6	19.03.2017	30.05.2017	72	19	0.26	371	112	20	4,48	230	184	0	0	0	0	0	0	0	0	0	I	
8	M	71	40	19.03.2017	11.06.2017	84	13	0.15	1,2	459	92	86,22	1,159	531	2	0	0	0.10	0.10	0	0	0.10	0.10	S	Forest on 12.05.2017
9	M	70	39.5	19.03.2017	20.07.2017	123	19	0.15	443	166	23	1,736	442	368	63	0.88	1.94	0.93	2.44	1.00	7.10	1.00	9.90	I	
10	M	70	33	19.03.2017	05.04.2017	17	7	0.41	236	92	34	1,219	218	150	0	0	0	0	0	0	0	0.05	0.05	S	
11	M	68	30,5	19.03.2017	08.04.2017	20	7	0.35	320	95	46	7,111	282	213	0	0	0	0.05	0.05	0	0	0.10	0.10	U	
12	M	70	39.1	19.03.2017	16.06.2017	89	15	0.17	532	166	35	18,44	466	174	1	0	0	0	0	0.59	0.59	0.59	0.59	S	
13	W	74	57	19.03.2017	27.05.2017	69	20	0.29	312	119	16	2095	241	219	1	0.06	0.06	0.06	0.06	0.34	0.34	0.41	0.44	S	
14	M	69	31.9	19.03.2017	26.05.2017	68	15	0.22	696	241	46	12,03	627	395	0	0	0	0.01	0.01	0	0	0.24	0.24	S	
15	W	80	50.5	19.03.2017	29.05.2017	71	10	0.14	109	38	11	722	122	91	0	0	0	0	0	0	0	0	0	S	
16	W	83	55.4	19.03.2017	31.05.2017	73	15	0.21	261	108	17	2	294	176	0	0	0	0	0	0	0	0	0	I	
17	M	70	32	19.03.2017	24.05.2017	66	12	0.18	261	67	22	6,639	94	42	13	0.12	0.12	0.12	0.12	0.19	0.20	0.19	0.20	P	
18	W	89	66.3	19.03.2017	06.06.2017	79	16	0.20	672	281	42	402	291	244	5	0.21	0.21	0.55	0.73	0.87	1.52	0.98	2.95	I	
19	W	86	64.4	19.03.2017	25.05.2017	67	17	0.25	907	439	53	11,88	777	691	16	0.12	0.12	0.12	0.12	0.31	0.35	0.31	0.35	P	
20	W	90	59.5	19.03.2017	20.03.2017	1	1	1.00	15	15	15	-	26	26	0	0	0	0	0	0	0	0	0	T	
21	M	72	38.7	20.03.2017	28.06.2017	100	12	0.12	367	135	31	3,686	170	84	1	0	0	0	0	0	0	0.08	0.08	I	
22	W	78	47.4	29.03.2017	18.05.2017	50	5	0.10	235	90	47	2,158	79	39	11	0	0	0	0	0.15	0.15	0.15	0.15	P	
23	M	76	38.9	02.05.2017	07.09.2017	128	28	0.22	448	95	17	4,228	191	128	36	0.66	0.99	0.75	1.24	1.00	7.75	1.00	8.88	E	
24	W	78	49.9	14.05.2017	20.06.2017	37	12	0.32	163	43	14	253	82	55	0	0	0	0	0	0	0	0	0	P	
25	W	80	55.6	19.05.2017	11.06.2017	23	11	0.48	213	128	19	1,977	164	134	1	0	0	0	0	0.79	0.79	0.79	0.79	A	
26	W	81	58.0	31.05.2017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	
27	W	78	45.0	04.06.2017	09.06.2017	5	4	0.80	35	20	9	10	24	21	0	0	0	0	0	0	0	0	0	S	
28	W	94	85.4	06.06.2017	18.06.2017	12	10	0.83	184	50	18	130	480	466	1	0.03	0.03	0.59	0.80	0.80	0.80	1.00	7.14	S	
29	W	80	55.0	12.06.2017	07.08.2017	56	16	0.29	529	68	33	2,194	367	317	10	0.66	0.95	0.66	0.95	1.00	6.06	1.00	6.06	P	
30	M	72	41.2	12.06.2017	27.06.2017	15	3	0.20	27	14	9	15	219	218	12	0.74	1.24	0.74	1.24	1.00	9.99	1.00	9.99	S	
31	M	76	40.6	13.06.2017	10.07.2017	27	5	0.19	445	243	89	3,673	350	207	18	0.91	2.09	0.91	2.09	1.00	13.79	1.00	13.79	P	
32	M	71	35.6	13.06.2017	05.07.2017	22	5	0.23	209	132	42	340	132	95	0	0	0	0	0	0	0	0	0	O	
33	W	82	56.8	15.06.2017	27.08.2017	73	32	0.44	1,3	336	39	85,46	1,018	399	28	0.87	1.79	0.92	2.23	1.00	15.69	1.00	18.45	P	Forest on 24.08.2017
34	M	77	38.8	15.06.2017	20.06.2017	5	2	0.40	59	38	29	-	416	406	0	0	0	0.05	0.05	0	0	0.80	0.80	S	

Indi	Sex	SVL [mm]	Weight [g]	Release date	Last relocation	Number of relocations	Different positions	AI	d_{total} [m]	d_{max} [m]	d_{mean} [m]	mcp_{95} [m²]	d_{pond_max} [m]	D_{pond_mean} [m]	Days in vineyard	exp_p application	exp_n application	exp_p application + spray drift	exp_n application + spray drift	exp_p application + DT_{50}	exp_n application + DT_{50}	exp_p application + spray drift + DT_{50}	exp_n application + spray drift + DT_{50}	Reason ending	Comment
35	M	80	52.3	15.06.2017	28.06.2017	13	2	0.15	186	146	93	-	232	222	0	0	0	0	0	0	0	0	0	P	
36	M	71	40.2	22.06.2017	13.07.2017	21	5	0.24	302	238	60	137	554	536	0	0	0	0	0	0	0	0	0	S	
37	W	93	58.9	22.06.2017	26.08.2017	65	12	0.18	1,1	185	96	7,348	183	147	3	0.02	0.02	0.02	0.02	0.76	1.02	0.93	1.72	I	
38	M	71	36.2	22.06.2017	25.06.2017	3	2	0.67	237	192	119	-	60	45	0	0	0	0	0	0	0	0	0	P	
39	W	84	51.3	27.06.2017	04.09.2017	69	20	0.29	660	115	33	3,566	373	323	24	0.89	1.89	0.90	1.99	1.00	14.34	1.00	15.86	A	
40	W	80	60.3	27.06.2017	11.07.2017	14	4	0.29	34	13	8	28	161	155	0	0	0	0	0	0	0	0	0	S	
41	M	70	32.1	07.07.2017	13.07.2017	6	1	0.17	18	18	18	-	4	4	0	0	0	0	0	0	0	0	0	P	
42	M	76	51.8	10.07.2017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	
43	M	72	45.1	10.07.2017	10.08.2017	31	4	0.13	160	48	40	374	255	240	30	0.94	2.52	0.94	2.52	1.00	20.70	1.00	20.70	I	
44	M	71	33.4	10.07.2017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	
45	M	72	32.8	10.07.2017	19.07.2017	9	3	0.33	614	362	205	2,593	972	944	1	0	0	0	0	0.60	0.60	0.60	0.60	S	Forest on 12.07.2017
46	M	76	49.9	21.07.2017	22.07.2017	1	1	1.00	7	7	7	-	153	153	0	0	0	0	0	0	0	0	0	S	
47	W	95	90.0	21.07.2017	26.07.2017	5	1	0.20	25	25	25	-	634	634	0	0	0	0	0	0	0	0	0	T	
48	W	98	99.1	21.07.2017	29.08.2017	39	8	0.21	304	74	38	1,72	287	255	0	0	0	0	0	0	0	0	0	I	
49	M	74	49.9	21.07.2017	05.09.2017	46	9	0.20	417	142	46	115	152	107	0	0	0	0	0	0	0	0	0	L	
50	M	79	46.9	27.07.2017	18.08.2017	22	10	0.45	772	250	77	11,17	275	195	0	0	0	0	0	0	0	0	0	L	
51	M	67	30.1	06.04.2017	11.05.2017	35	9	0.26	56	9	6	76	66	62	0	0	0	0	0	0	0	0	0	A	
52	M	83	69.1	16.10.2017	24.10.2017	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	Forest on 24.10.2017
53	W	69	35.0	16.10.2017	08.11.2017	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	
54	M	70	33.7	16.10.2017	24.10.2017	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	
55	M	94	81.3	07.11.2017	22.11.2017	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	Forest on 16.11.2017
56	W	96	72.9	07.11.2017	22.11.2017	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	
57	W	72	44.7	07.11.2017	08.11.2017	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	

Appendix C. Detailed information about individuals and pesticide applications per day.

Details about the part of the population that is expected within the agricultural area (Pleftd), the number of tracked toads ntd, the number toads located in vineyards (nvd; with and without considering spray drift), the probability that a vineyard was treated with a pesticide (pd; with and without considering the DT50) and the expected part of the population that was exposed to pesticides (popd; for all four scenarios) for each day.

Date	Pleft_d	nt_d	nv_d	nv_d + spray drift	p_d	p_d + DT_{50}	pop_d	pop_d + spray drift	pop_d + DT_{50}	pop_d + spray drift + DT_{50}
19.03.2017	0.996	2	0	0	0	0	0	0	0	0
20.03.2017	0.992	17	2	3	0	0	0	0	0	0
21.03.2017	0.988	17	3	6	0	0	0	0	0	0
22.03.2017	0.984	17	4	5	0	0	0	0	0	0
23.03.2017	0.980	17	3	5	0	0	0	0	0	0
24.03.2017	0.976	17	3	6	0	0	0	0	0	0
25.03.2017	0.972	17	3	5	0	0	0	0	0	0
26.03.2017	0.968	17	3	5	0	0	0	0	0	0
27.03.2017	0.964	17	3	5	0	0	0	0	0	0
28.03.2017	0.960	16	3	5	0	0	0	0	0	0
29.03.2017	0.956	16	3	5	0.01	0.01	0.18	0.30	0.18	0.30
30.03.2017	0.952	17	3	5	0.01	0.02	0.17	0.28	0.34	0.56
31.03.2017	0.948	17	3	5	0.05	0.07	0.84	1.39	1.17	1.95
01.04.2017	0.944	17	3	6	0.05	0.10	0.83	1.67	1.67	3.33
02.04.2017	0.940	17	4	7	0	0.10	0	0	2.21	3.87
03.04.2017	0.936	17	4	7	0	0.05	0	0	1.10	1.93
04.04.2017	0.932	17	3	6	0	0	0	0	0	0
05.04.2017	0.928	17	2	3	0	0	0	0	0	0
06.04.2017	0.924	16	3	4	0	0	0	0	0	0
07.04.2017	0.920	17	2	3	0	0	0	0	0	0
08.04.2017	0.916	17	2	3	0	0	0	0	0	0
09.04.2017	0.912	16	2	3	0	0	0	0	0	0
10.04.2017	0.908	16	2	3	0	0	0	0	0	0
11.04.2017	0.904	16	2	3	0	0	0	0	0	0
12.04.2017	0.900	16	2	3	0	0	0	0	0	0
13.04.2017	0.896	16	1	2	0.08	0.08	0.45	0.90	0.45	0.90
14.04.2017	0.892	16	1	2	0	0.08	0	0	0.45	0.89
15.04.2017	0.888	16	1	2	0	0.08	0	0	0.44	0.89
16.04.2017	0.884	16	1	1	0	0	0	0	0	0
17.04.2017	0.880	16	1	1	0	0	0	0	0	0
18.04.2017	0.876	16	1	1	0	0	0	0	0	0
19.04.2017	0.871	16	1	1	0	0	0	0	0	0
20.04.2017	0.867	16	1	1	0	0	0	0	0	0
21.04.2017	0.863	16	1	1	0	0	0	0	0	0
22.04.2017	0.859	16	1	1	0	0	0	0	0	0
23.04.2017	0.855	16	1	1	0	0	0	0	0	0
24.04.2017	0.851	16	1	1	0	0	0	0	0	0
25.04.2017	0.847	16	1	1	0	0	0	0	0	0
26.04.2017	0.843	16	1	1	0	0	0	0	0	0
27.04.2017	0.839	16	1	1	0.10	0.10	0.52	0.52	0.52	0.52
28.04.2017	0.835	16	1	1	0	0.10	0	0	0.52	0.52
29.04.2017	0.831	16	1	1	0	0.10	0	0	0.52	0.52
30.04.2017	0.827	16	1	1	0	0	0	0	0	0
01.05.2017	0.823	16	1	1	0	0	0	0	0	0
02.05.2017	0.819	16	1	1	0	0	0	0	0	0
03.05.2017	0.815	17	1	2	0.20	0.20	0.94	1.87	0.94	1.87

Date	$Pleft_d$	nt_d	nv_d	nv_d + spray drift	p_d	p_d + DT_{50}	pop_d	pop_d + spray drift	pop_d + DT_{50}	pop_d + spray drift + DT_{50}
04.05.2017	0.811	17	1	2	0	0.20	0	0	0.93	1.86
05.05.2017	0.807	17	1	2	0	0.20	0	0	0.93	1.85
06.05.2017	0.803	17	1	2	0	0	0	0	0	0
07.05.2017	0.799	17	2	3	0	0	0	0	0	0
08.05.2017	0.795	17	4	5	0	0	0	0	0	0
09.05.2017	0.791	17	3	5	0.10	0.10	1.40	2.33	1.40	2.33
10.05.2017	0.787	17	3	5	0.01	0.11	0.20	0.33	1.59	2.65
11.05.2017	0.783	17	2	4	0.11	0.23	1.05	2.11	2.11	4.21
12.05.2017	0.779	16	1	2	0.16	0.29	0.80	1.60	1.43	2.85
13.05.2017	0.775	16	2	3	0.06	0.34	0.62	0.93	3.32	4.98
14.05.2017	0.771	16	1	3	0.01	0.24	0.07	0.21	1.17	3.51
15.05.2017	0.767	17	2	3	0.21	0.29	1.89	2.83	2.60	3.90
16.05.2017	0.763	17	1	3	0.11	0.34	0.51	1.54	1.52	4.55
17.05.2017	0.759	17	1	2	0.05	0.37	0.22	0.45	1.67	3.34
18.05.2017	0.755	17	0	1	0.15	0.31	0	0.67	0	1.40
19.05.2017	0.751	16	1	4	0.10	0.30	0.47	1.88	1.41	5.63
20.05.2017	0.747	17	0	2	0	0.25	0	0	0	2.20
21.05.2017	0.743	17	0	2	0	0.10	0	0	0	0.87
22.05.2017	0.739	17	0	2	0.10	0.10	0	0.87	0	0.87
23.05.2017	0.735	17	0	2	0.40	0.50	0	3.46	0	4.32
24.05.2017	0.731	17	0	1	0.20	0.70	0	0.86	0	3.01
25.05.2017	0.727	16	0	1	0	0.60	0	0	0	2.73
26.05.2017	0.723	15	0	1	0.10	0.40	0	0.48	0	1.93
27.05.2017	0.719	14	0	1	0	0.20	0	0	0	1.03
28.05.2017	0.715	13	0	1	0	0.20	0	0	0	1.10
29.05.2017	0.711	13	0	1	0.15	0.25	0	0.82	0	1.37
30.05.2017	0.707	12	0	1	0.05	0.30	0	0.29	0	1.77
31.05.2017	0.703	11	0	1	0	0.30	0	0	0	1.92
01.06.2017	0.699	10	0	1	0.25	0.30	0	1.75	0	2.10
02.06.2017	0.695	10	0	1	0.15	0.40	0	1.04	0	2.78
03.06.2017	0.691	10	0	1	0.19	0.59	0	1.31	0	4.08
04.06.2017	0.687	10	2	3	0	0.59	0	0	8.10	12.16
05.06.2017	0.683	11	3	3	0	0.49	0	0	9.12	9.12
06.06.2017	0.679	11	2	3	0.10	0.59	1.23	1.85	7.28	10.92
07.06.2017	0.675	11	2	3	0.10	0.59	1.23	1.84	7.24	10.86
08.06.2017	0.671	11	2	3	0.30	0.84	3.66	5.49	10.24	15.36
09.06.2017	0.667	11	2	3	0.10	0.79	1.21	1.82	9.58	14.36
10.06.2017	0.663	10	1	3	0	0.79	0	0	5.23	15.70
11.06.2017	0.659	10	2	4	0	0.79	0	0	10.41	20.81
12.06.2017	0.655	8	1	3	0.10	0.89	0.82	2.45	7.28	21.85
13.06.2017	0.651	10	1	3	0.30	0.90	1.95	5.86	5.86	17.57
14.06.2017	0.647	12	2	4	0.15	0.90	1.56	3.13	9.70	19.40
15.06.2017	0.643	12	1	2	0	0.80	0	0	4.28	8.57
16.06.2017	0.639	15	3	7	0.05	0.80	0.64	1.49	10.22	23.84
17.06.2017	0.635	14	3	5	0.13	0.80	1.70	2.83	10.81	18.02
18.06.2017	0.631	14	4	4	0.03	0.80	0.45	0.45	14.32	14.32
19.06.2017	0.627	13	3	3	0.18	0.90	2.53	2.53	12.94	12.94
20.06.2017	0.622	13	2	2	0.08	0.90	0.72	0.72	8.57	8.57
21.06.2017	0.618	11	2	2	0	0.90	0	0	10.06	10.06
22.06.2017	0.614	11	2	2	0.25	0.87	2.79	2.79	9.72	9.72
23.06.2017	0.610	14	2	2	0.14	0.80	1.22	1.22	6.93	6.93
24.06.2017	0.606	14	2	2	0	0.80	0	0	6.89	6.89
25.06.2017	0.602	14	2	2	0.10	0.80	0.82	0.82	6.88	6.88
26.06.2017	0.598	13	3	3	0.15	0.85	2.07	2.07	11.74	11.74
27.06.2017	0.594	13	2	2	0.15	0.80	1.37	1.37	7.32	7.32
28.06.2017	0.590	14	1	2	0	0.70	0	0	2.95	5.90

Date	Pleft$_d$	nt$_d$	nv$_d$	nv$_d$ + spray drift	p$_d$	p$_d$ + DT$_{50}$	pop$_d$	pop$_d$ + spray drift	pop$_d$ + DT$_{50}$	pop$_d$ + spray drift + DT$_{50}$
29.06.2017	0.586	12	1	1	0	0.60	0	0	2.93	2.93
30.06.2017	0.582	12	1	1	0	0.60	0	0	2.91	2.91
01.07.2017	0.578	12	3	3	0	0.50	0	0	7.23	7.23
02.07.2017	0.574	12	3	3	0	0.50	0	0	7.18	7.18
03.07.2017	0.570	12	2	2	0.40	0.65	3.80	3.80	6.18	6.18
04.07.2017	0.566	12	2	2	0.20	0.70	1.89	1.89	6.61	6.61
05.07.2017	0.562	12	2	2	0	0.70	0	0	6.56	6.56
06.07.2017	0.558	11	1	1	0.25	0.75	1.27	1.27	3.81	3.81
07.07.2017	0.554	11	0	0	0.15	0.70	0	0	0	0
08.07.2017	0.550	12	0	0	0	0.70	0	0	0	0
09.07.2017	0.546	12	0	0	0	0.70	0	0	0	0
10.07.2017	0.542	12	0	0	0	0.65	0	0	0	0
11.07.2017	0.538	13	4	4	0	0.60	0	0	9.94	9.94
12.07.2017	0.534	12	3	3	0.05	0.60	0.67	0.67	8.01	8.01
13.07.2017	0.530	12	3	3	0.25	0.80	3.31	3.31	10.60	10.60
14.07.2017	0.526	10	3	3	0.05	0.80	0.79	0.79	12.63	12.63
15.07.2017	0.522	10	2	2	0.05	0.80	0.52	0.52	8.35	8.35
16.07.2017	0.518	10	2	2	0	0.70	0	0	7.25	7.25
17.07.2017	0.514	10	2	2	0.40	0.80	4.11	4.11	8.22	8.22
18.07.2017	0.510	10	1	1	0.10	0.80	0.51	0.51	4.08	4.08
19.07.2017	0.506	10	1	1	0	0.80	0	0	4.05	4.05
20.07.2017	0.502	9	1	1	0	0.60	0	0	3.35	3.35
21.07.2017	0.498	7	2	2	0	0.50	0	0	7.11	7.11
22.07.2017	0.494	11	5	5	0	0.50	0	0	11.23	11.23
23.07.2017	0.490	10	5	6	0	0.50	0	0	12.25	14.70
24.07.2017	0.486	10	5	6	0	0.50	0	0	12.15	14.58
25.07.2017	0.482	10	5	6	0	0.50	0	0	12.05	14.46
26.07.2017	0.478	10	6	6	0.03	0.48	0.96	0.96	13.86	13.86
27.07.2017	0.474	9	6	6	0.28	0.57	8.95	8.95	17.90	17.90
28.07.2017	0.470	10	5	6	0.25	0.63	5.95	7.14	14.68	17.62
29.07.2017	0.466	10	5	5	0.21	0.75	4.78	4.78	17.39	17.39
30.07.2017	0.462	10	4	4	0.03	0.74	0.46	0.46	13.64	13.64
31.07.2017	0.458	10	2	2	0.18	0.85	1.60	1.60	7.78	7.78
01.08.2017	0.454	10	2	3	0.15	0.78	1.33	1.99	7.03	10.55
02.08.2017	0.450	10	3	3	0	0.75	0	0	10.12	10.12
03.08.2017	0.446	10	2	4	0	0.68	0	0	6.02	12.04
04.08.2017	0.442	10	2	4	0.05	0.68	0.44	0.88	5.96	11.93
05.08.2017	0.438	10	3	4	0.15	0.78	1.97	2.63	10.18	13.57
06.08.2017	0.434	10	3	4	0	0.78	0	0	10.08	13.45
07.08.2017	0.430	10	2	4	0.28	0.88	2.41	4.81	7.56	15.13
08.08.2017	0.426	9	1	3	0	0.78	0	0	3.69	11.07
09.08.2017	0.422	9	1	3	0.02	0.80	0.09	0.28	3.75	11.24
10.08.2017	0.418	9	4	4	0.05	0.50	0.84	0.84	9.28	9.28
11.08.2017	0.414	8	4	4	0.13	0.50	2.59	2.59	10.34	10.34
12.08.2017	0.410	8	4	4	0	0.45	0	0	9.22	9.22
13.08.2017	0.406	8	4	4	0	0.45	0	0	9.13	9.13
14.08.2017	0.402	8	3	4	0.10	0.50	1.51	2.01	7.47	9.96
15.08.2017	0.398	8	3	4	0	0.45	0	0	6.71	8.95
16.08.2017	0.394	8	3	3	0	0.45	0	0	6.64	6.64
17.08.2017	0.390	8	2	3	0	0.35	0	0	3.41	5.11
18.08.2017	0.386	8	2	3	0	0.30	0	0	2.89	4.34
19.08.2017	0.382	7	2	3	0	0.25	0	0	2.73	4.09
20.08.2017	0.378	7	1	3	0.01	0.26	0.05	0.15	1.40	4.19
21.08.2017	0.373	7	1	3	0	0.17	0	0	0.91	2.72
22.08.2017	0.369	7	1	3	0	0.17	0	0	0.90	2.69
23.08.2017	0.365	7	2	3	0	0.17	0	0	1.78	2.66

Date	$Pleft_d$	nt_d	nv_d	nv_d + spray drift	p_d	p_d + DT_{50}	pop_d	pop_d + spray drift	pop_d + DT_{50}	pop_d + spray drift + DT_{50}
24.08.2017	0.361	7	1	2	0	0.13	0	0	0.65	1.29
25.08.2017	0.357	7	2	3	0.02	0.02	0.20	0.31	0.20	0.31
26.08.2017	0.353	7	1	2	0	0.02	0	0	0.10	0.20
27.08.2017	0.349	6	1	2	0	0.02	0	0	0.12	0.23
28.08.2017	0.345	5	1	2	0.02	0.02	0.14	0.28	0.14	0.28
29.08.2017	0.341	5	1	2	0	0.02	0	0	0.14	0.27
30.08.2017	0.337	4	1	2	0.03	0.05	0.26	0.52	0.43	0.86
31.08.2017	0.333	4	1	2	0	0	0	0	0	0
01.09.2017	0.329	4	1	2	0	0	0	0	0	0
02.09.2017	0.325	4	1	2	0	0	0	0	0	0
03.09.2017	0.321	4	1	2	0	0	0	0	0	0
04.09.2017	0.317	4	1	2	0	0	0	0	0	0
05.09.2017	0.313	3	1	1	0	0	0	0	0	0
06.09.2017	0.309	2	1	1	0	0	0	0	0	0
07.09.2017	0.305	2	1	1	0	0	0	0	0	0

Chapter 2

Avoidance behavior of juvenile common toads (*Bufo bufo*) in response to surface contamination by different pesticides

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Abstract

Most agricultural soils are expected to be contaminated with agricultural chemicals. As the exposure to pesticides can have adverse effects on non-target organisms, avoiding contami- nated areas would be advantageous on an individual level, but could lead to a chemical land- scape fragmentation with disadvantages on the metapopulation level. We investigated the avoidance behavior of juvenile common toads (*Bufo bufo*) in response to seven pesticide for- mulations commonly used in German vineyards. We used test arenas filled with silica sand and oversprayed half of each with different pesticide formulations. We placed a toad in the middle of an arena, filmed its behavior over 24 hours, calculated the proportion of time a toad spent on the contaminated side and compared it to a random side choice. We found evidence for the avoidance of the folpet formulation Folpan® 500 SC, the metrafenone formulation Vivando® and the glyphosate formulation Taifun® forte at maximum recommended field rates for vine and a trend for avoidance of Wettable Sulphur Stulln (sulphur). No avoidance was observed when testing Folpan® 80 WDG (folpet), Funguran® progress (copper hydrox- ide), SpinTorTM (spinosad), or 10% of the maximum field rate of any formulation tested. In the choice-tests in which we observed an avoidance, toads also showed higher activity on the contaminated side of the arena. As video analysis with tracking software is not always feasi- ble, we further tested the effect of reducing the sampling interval for manual data analyses. We showed that one data point every 15 or 60 minutes results in a risk of overlooking a weak avoidance behavior, but still allows to verify the absence/presence of an avoidance for six out of seven formulations. Our findings are important for an upcoming pesticide risk assessment for amphibians and could be a template for future standardized tests.

Introduction

About 40% of the area of the European Union is agriculturally used [1], making agriculture the dominant type of landscape in many regions. Modern agriculture is often linked to extensive use of agrochemicals to maximize crop yield. In 2017, 327 million kg of herbicides, insecticides and fungicides were sold in the EU to control pests, weeds, and diseases in agricultural fields [2]. This results in a contamination of most agricultural topsoils with pesticides [3, 4]. As breeding ponds of European amphibians can often be found within or near crops [5–7], amphibians are likely to come in contact with pesticides and contaminated soils during their pre- or post-breeding migration [8–10] possibly resulting in an uptake of pesticides [11, 12]. As the exposure to pesticides can have sublethal [13–15] and even lethal [16–18] effects, physiological and behavioral adaptations of amphibians to pesticides would decrease the hazard. Indeed, several studies found evidence for evolved pesticide tolerance in terms of decreased sensitivity in amphibian larvae of populations frequently exposed to pesticides, e.g. in *Lithobates sylvaticus* [19] or *Rana temporaria* [20]. The simplest behavioral response to minimize adverse effects might be to avoid a contamination. Such a response presupposes that amphibians are able to sense it.

Amphibians have good olfactory perception [21, 22], and a pesticide-permeable skin [23] allowing the uptake of large molecules [24]. Additionally, some pesticides used in agriculture are considered to be skin-irritating for humans, which is most likely also true for amphibians. Therefore, amphibians might be able to perceive contaminations and to assess the quality and suitability of water and surfaces to avoid them [25]. Several mesocosm and laboratory experiments investigated the avoidance of contaminated water bodies [26–28] as well as surfaces like soil or filter paper [11, 29–32]. Results are partly contradictory and might depend on the species, the substrate, the exposure period, the contaminant, and its concentration. Field studies that support surface laboratory tests are scarce, but some showed that amphibians tend to avoid arable fields as habitat and prefer non-cultivated areas [8, 33, 34]. Also genetic studies suggested a barrier effect of agricultural fields [35, 36]. However, it remains unknown if these effects are partly caused by pesticides or if they are solely the results of habitat characteristics.

For European amphibian species, studies on the avoidance of contaminated surfaces are lacking. Therefore, in the present study, we investigated the avoidance behavior of the common toad (*Bufo bufo* Linnaeus, 1758) in response to surface contamination by seven different pesticide formulations. We performed a laboratory experiment in which juvenile toads could choose between a contaminated and an uncontaminated side of a test arena. In general, our setup is comparable with those used in previous studies [11, 29–31], but instead of determining the side choice in intervals of minutes to hours, we continuously filmed the behavior of a toad in the arena over 24 h. Based on this video material, we answered the question if *B. bufo* avoids surfaces that had been contaminated with pesticides at 100% and 10% of the maximum recommended field rate. Continuous filming requires specialized hardware and, as it results in hundreds of hours of videos, also specialized tracking software to analyze the data. This comes with limitations in the experimental setup, e.g. the contrast between the surface and the experimental animal has to be high enough to allow a reliable tracking. Therefore, we tested if a reduced data set, which would also allow a manual analysis, results in the same pattern of potential avoidance behavior. As alterations of the movement behavior after pesticide exposure are well known for amphibian larvae [37], we further tested if the toads exhibit a different activity on the contaminated side of the arena.

Material and methods

Study species, sampling and animal husbandry

The common toad (*Bufo bufo* Linnaeus, 1758) is one of the most widespread amphibian species in Europe [38] and can be found in ponds within or near vineyards [6]. *Bufo bufo* is listed as "least concern" by the IUCN [39], but there are local declines of populations in their entire distribution area [40–43]. Although there is a trend to avoid vineyards as habitat, adult toads can be found directly in vineyards during their post-breeding migration and their risk for coming in contact with contaminated soil is high [8]. To investigate the potential of avoiding contaminated soils, we used juvenile toads because they are leaving their aquatic habitat between May and August in Germany [44], a time when most pesticides are applied in vineyards [8]. Further, juveniles play an important role in the dispersal and the population connectivity in many amphibian species [45]. Thus, an avoidance behavior of juveniles might have particularly adverse effects on the connectivity of populations.

Between the end of July and mid-September 2018 (see S1 Table for exact dates), juveniles of *B*. *bufo* (about 10 to 20 mm; metamorphed in June) were caught next to a permanent rainwater retention pond near Siebeldingen (Rhineland-Palatinate, Germany; 49.218368 N, 8.049538 E (WSG84); 196 m asl; S1 Fig). As the pond is used by hundreds of breeding individuals each year, we expect that the juveniles are from several different clutches. The pond is surrounded by a vegetative buffer strip, but is located in a landscape dominated by vineyards. As viticulture is a pesticide intensive crop with on average 9.5 pesticide applications per year in Germany [8, 46], the pond and the soils in the nearby vineyards can be expected to be contaminated with various agrochemicals. Thus, also toads using this pond can be expected to be regularly exposed to pesticides, both during their aquatic and terrestrial life stages. Collected toads were kept in groups of up to 40 individuals in outdoor net cages (40 x 65 x 30 cm) between six and 15 days (mean = 9.8 ± 4.3 days; see S1 Table for exact time spans) before an experiment. Individuals for the last choice-test (Wettable Sulphur Stulln) were only kept for one day. Cages were equipped with about 5 cm soil, moss and leaves as hiding places and were regularly watered with untreated tap water. Soil, moss and leaves were collected in the Palatinate Forest in a distance of about 1.6 km to the nearest vineyard and were therefore expected to be not contaminated with pesticides (S1 Fig). Toads were fed *ad libitum* with *Drosophila sp*. (own breed or purchased in a pet shop) or small insects ("meadow plankton") caught on a meadow where no pesticides are used (distance to the nearest vineyard = 2 km; S1 Fig). The day before an experimental run, animals were weighed to the nearest mg (CP153; Sartorius AG, Göttingen, Germany; see S1 Table for the mean weight of the individuals per experimental run), transferred into plastic boxes (11.5 x 17.5 x 13 cm) filled with about 2 cm of moist soil, moss and leaves and kept individually in the laboratory until the experiment. During this time the toads were not fed. Individuals chosen for an experimental run had been kept in the outdoor cages over the same time period. Further, we aimed to minimize the variance of the body weight within an experimental run. As common toads are explosive breeders we expected all individuals to have a similar age.

Ethics statement

The study was approved by the Landesuntersuchungsamt in Koblenz (Germany; approval number G17-20-044). The collection of toads was permitted by the"Struktur- und Genehmigungsdirektion Süd Referat 42—Obere Naturschutzbehörde" (Neustadt an der Weinstraße, Germany; approval number 42/553-254/ 457-18(1)).

Test substances

Experiments were performed with one insecticide, one herbicide and five different fungicide formulations (Table 1) that are frequently used in German vineyards and also in the area around the pond where toads were captured [8]. Commercial pesticides were obtained from a local distributor and the Julius Kühn-Institut (Siebeldingen, Germany). Three of the pesticide formulations are also approved for organic farming (Table 1). For each pesticide, the maximum recommended field rate (FR_{max}) for vine was used. For four pesticides the test was also conducted with 10% of FR_{max}. As we were limited in the number of performed test runs and most vineyards are managed conventionally, we tested only the conventional pesticides (Folpan® 500 SC, Folpan® 80 WDG, Taifun® forte, and Vivando®) with 10% of FR_{max}. All stock solutions were prepared with tap water according to the manner of a common user for a water application rate of 200 L/ha.

Experimental setup

All experiments were performed in glass petri dishes with a diameter of 20 cm filled with 300 g silica sand (SILIGRAN® dry, grain size: 0.1–0.3 mm; Euroquarz GmbH, Dorsten, Germany). We chose a bright sand to enhance the visual contrast of toads and background for subsequent filming. Prior to pesticide application, the sand was moistened with 29.85 mL of tap water (equivalent to 9,500 L/ha) by using a laboratory spray application system (Schachtner, Ludwigsburg, Germany). One half of each test arena was covered with a laminated paper semicircle (S2 Fig), and the pesticide stock solution was applied with the application system and an application rate of 200 L/ha. This resulted in a split design, with exactly one half of each test arena uncontaminated and one half contaminated with 0.31 ml of the pesticide solution. As the amount of pesticide is only about 2% of the amount of applied water, we neglected the resulting differences in the moisture between the two sides and did not apply additional water on the uncontaminated side. The test arena walls were then shielded with white paper strips to minimize external cues for the toads. To prevent escaping but still allow gas exchange and filming of the toads, each arena was covered with a polyamide fabric (sheer tights with 8 denier).

Table 1: Pesticide formulations used for choice-tests with their maximum recommended field rate (FR_{max}) for vine and the contained amount of active ingredient (A.I.).

Formulation	Type	A.I.	FR_{max} formulation	FR_{max} A.I.	Organic farming	CLP-Classification [1]
Folpan® 500 SC [2]	Fungicide	Folpet	2.4 L/ha	1.2 kg/ha	No	H315, H317
Folpan® 80 WDG [2]	Fungicide	Folpet	1.6 kg/ha	1.28 kg/ha	No	H317
Funguran® progress [3]	Fungicide	Copper hydroxide	2 kg/ha	1.074 kg/ha	Yes	-
SpinTor™ [4]	Insecticide	Spinosad	160 mL/ha	76.8 g/ha	Yes	-
Taifun® forte [2]	Herbicide	Glyphosate	5 L/ha	1.8 kg/ha	No	H314
Vivando® [5]	Fungicide	Metrafenone	320 mL/ha	160 g/ha	No	H317, H315
Wettable Sulphur Stulln [6]	Fungicide	Sulphur	3.2 kg/ha	2.55 kg/ha	Yes	H315

[1] At least the A.I. or one of the additives is classified according to Regulation (EC) No. 1272/2008 [CLP] as "Causes severe skin burns and eye damage" (H314), "Causes skin irritation" (H315), "May cause an allergic skin reaction" (H317). Other classifications that are not related to the skin were not considered.
[2] ADAMA Deutschland GmbH; Cologne, Germany
[3] Spiess-Urania Chemicals GmbH; Hamburg, Germany
[4] DowDuPont Inc.; Wilmington, USA
[5] BASF SE; Ludwigshafen am Rhein, Germany
[6] Agrostulln GmbH; Stulln, Germany

For one experimental run (i.e. one pesticide at one concentration; S1 Table) 16 replicates (i.e. 16 test arenas with one toad each; resulting in a total of 192 toads over the whole study) were used. Two arenas were placed in one dark test chamber (S3 Fig). The contaminated side of the arena was orientated randomly into one of the cardinal directions. An LED light was attached above each arena for illumination without shading the arena. A camera system, consisting of a Raspberry Pi (Raspberry Pi 3 Model B; Raspberry Pi Foundation, Cambridge, UK) with a camera module (SC15; Kuman Ltd., Shenzhen, China; S4 Fig) was attached to each test chamber. The camera was facing upside down to allow the filming of two arenas at the same time (S5 Fig). Videos were taken with a resolution of 1,296 x 730 pixels and 24 frames per second and saved as 30 or 60 min long H.264 files.

At latest 90 min after the application of the pesticides, one toad was placed in the center of a test arena and filming started for 24 h. The light was automatically turned off at 10 pm (about 10 h after test initiation) for 8 h. During this time, the arenas were illuminated with IR-light, which cannot be sensed by *B. bufo*, but allows continuous filming. Neither the test chambers nor the room with the test chambers had a sound insulation, but the room was not entered during any experimental run. Temperature during filming was 23 ± 2°C and the humidity between 57 and 81%. The toads were not fed during the time of the experimental run and were released in a distance of 200 m to the pond after the run.

Before the choice-tests with seven different pesticides, we conducted one control-test in completely uncontaminated arenas (n = 16) to exclude the presence of any external influences on the side choice or a preference for a cardinal direction.

Video analysis

The recorded videos of the choice-tests were converted into MP4 files with the software XMedia Recode (Version 3.4.5.0; Sebastian Dörfler, Günthersleben-Wechmar, Germany). The software EthoVision® XT (Version 12.0; Noldus Information Technology, Wageningen, Netherlands) was used to track the toads in the arenas. Toads were extracted from the background via dynamic and static subtraction. EthoVision® XT determined every 0.4167 seconds (= sampling interval) if a toad was sitting in predefined zones within the arena (matching the contaminated and the uncontaminated side). Positions within a 2.5 cm wide area at the border between both sides (buffer zone) were excluded to take possible inaccuracies and unintended contaminations during the application process or leakage of the pesticide into account (see S1 Table for the mean time in the buffer zone per experimental run). Additionally, the distance moved between two time points was calculated. To reduce noise in the acquired tracks, track smoothing with a 2 mm threshold was used (method "minimal distance moved" with "direct" option in EthoVision® XT). Tracks were checked for errors and reanalyzed with adjusted settings when necessary. Videos of the control-test were analyzed in the same way, but each arena was divided into halves orientated to the north & south and to the east & west.

Parameters evaluated and statistical analysis

For statistical analysis, raw data from EthoVision® XT were exported to R, version 3.4.3 [48]. To allow an acclimatization of the toads in the arenas, video material from the first three minutes of an experimental run were skipped during the analysis in EthoVision® XT. Data from the following 12 minutes were excluded during the data analysis in R, resulting in a total acclimatization period of 15 minutes. For choice-tests, the percentage of time (t) an individual spent on the contaminated side of an arena (t_{pest}) was calculated. To analyze if a reduction of the sampling interval affects the probability to detect an avoidance behavior, we subsampled the 24 hours of raw data and recalculated t_{pest} based on a sampling interval of 10 seconds (t_{pest_10}), 60 seconds (t_{pest_60}), 15 minutes (900 seconds, t_{pest_900}) and 60 minutes (3,600 seconds, t_{pest_3600}), starting with the first data point after the acclimatization period. Additionally, we reduced our data to the first hour of a choice-test (t_{pest_1h}) without changing the sampling interval and thus ignored the remaining 23 hours of an experimental run. For the control-test, t was calculated for the side orientated to the north (t_{north}) and west (t_{west}). To identify a possible bias caused by the position of the arena within a test chamber or of the test chamber within the room, we calculated t also for the side of the arena orientated to the wall of the room (t_{wall}) and to the second arena in the chamber (t_{arena}). Both the direction to the wall and to the second arena correspond to a cardinal direction. Following the approach of Hatch et al. [29] and Gertzog et al. [31], two-sided one-sample Wilcoxon signed-rank tests were used to compare t to a theoretical value of 50% that can be expected from a random side choice for each experimental run. Additionally, two-sided paired Wilcoxon signed-rank tests were used to test for differences between t_{pest} and t_{pest_10}, t_{pest_60}, t_{pest_900}, t_{pest_3600} or t_{pest_1h}.

As additional behavioral endpoint for the choice-tests, the total distance moved per side (d) was calculated as measure of toad activity. To enable a comparison between moved distances on contaminated (d_{pest}) and uncontaminated (d_{clean}) sides, distances were corrected for the respective time spent per side and are given in meters per hour. As distances were not normally distributed, we used two-sided paired Wilcoxon signed-rank tests to test for differences between d_{pest} and d_{clean}.

For all statistical tests, the criterion for significance was 0.05. When testing t_{pest} against 50% or d_{pest} against d_{clean}, p-values from all tested formulations with the same concentration (n = 7 for 100% of FR_{max}, n = 4 for 10% of FR_{max}) were adjusted (p adj.) using the false discovery rate (FDR) method described by Benjamini and Hochberg [49]. As we wanted to see if the subsampling of the data would lead to the same avoidance pattern in a screening of the seven tested pesticide formulations, we also used FDR to adjust the p-values when testing t_{pest} against t_{pest_10}, t_{pest_60}, t_{pest_900}, t_{pest_3600} and t_{pest_1h} in the same way. However, as we were also interested if the subsampling results in differences independent of the number of tested formulations in the screening, we also presented unadjusted p-values. P-values of the control-test and when testing t_{pest} against t_{pest_10}, t_{pest_60}, t_{pest_900}, t_{pest_3600} or t_{pest_1h} were also not adjusted. Median values ($\tilde{t}$) are given with their interquartile range (IQR).

Results

The control-test revealed neither a preference for any cardinal direction ($\tilde{t}_{north}$ = 49.2%, IQR = 30.0–71.9%; Wilcoxon test vs. 50%: V = 72, p = 0.860; $\tilde{t}_{west}$ = 51.2%, 29.9–61.9%; V = 74, p = 0.782; n = 16 in all tests) nor for the side orientated to the wall ($\tilde{t}_{wall}$ = 39.4%, 26.6–69.6%; V = 63, p = 0.821) or to the other arena ($\tilde{t}_{arena}$ = 41.9, 28.7–52.2%; V = 44, p = 0.231) over 24 hours.

The animals spent on average less than 50% of their time on the contaminated side of the arena in all tested formulations at FR_{max} ($\tilde{t}_{pest}$ < 50%; Fig 1 and Table 2), with the exception of Funguran® progress and Folpan® 80 WDG. Avoidance was significant for Folpan® 500 SC, Vivando® and Taifun® forte (Table 2). There was also a trend to avoid the contaminated side for Wettable Sulphur Stulln (p adj. = 0.068, but p = 0.039 without FDR; Table 2). No significant avoidance was observed when using a concentration of 10% of FR_{max} in any formulation (Table 2).

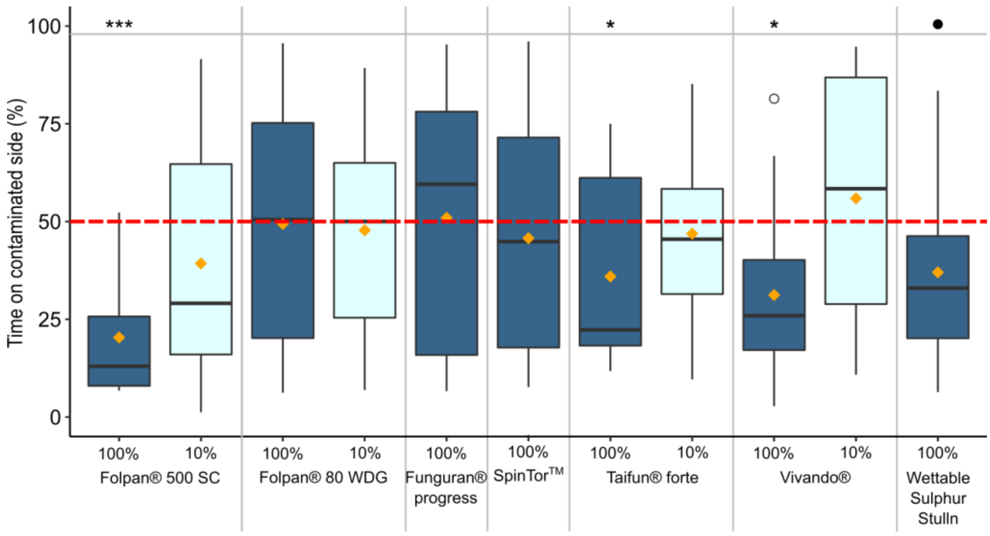

Fig. 1: Boxplots showing the proportion of time a toad spent on the contaminated side of an arena over 24 hours for each tested formulation and concentration (t_{pest} in percentage; dark blue = 100% of the maximum recommended field rate (FR_{max}), light blue = 10% of FR_{max}). In each boxplot, the boundaries of the box are the 25th and 75th percentiles and the whiskers correspondent to the lowest and largest value no further than 1.5 times from the 25th and 75th percentiles away. Data points beyond the whiskers are shown as unfilled circles. Median values are presented as horizontal lines and orange diamonds show the mean values. Significant difference from a random choice (50%; red dotted line): ●: p adj. < 0.1; *: p adj. < 0.05; ***: p adj. < 0.001. P-values from tests with the same concentration were adjusted using the FDR. N = 16 per choice-test.

Table 2: Proportion of time a toad spent on the contaminated side of an arena (t_{pest}) for each tested formulation and concentration (10% or 100% of the maximum recommended field rate; FR_{max}) and results from two-sided one-sample Wilcoxon signed-rank tests that were used to compare *tpest* to a theoretical value of 50% that can be expected from a random side choice.

Formulation	**% of FR_{max}**	**Time on contaminated side (%) t_{pest}** Median	**IQR**	**Range**	**Mean**	**Wilcoxon-Test - compared to 50%** V	**p**	**p adj.**
Folpan® 500 SC	100	13.0	8.0 - 25.7	6.8 - 52.3	20.3	2	**< 0.001**	**< 0.001**
Folpan® 500 SC	10	29.1	16.0 - 64.7	1.2 - 91.6	39.3	41	0.175	0.701
Folpan® 80 WDG	100	50.5	20.2 - 75.2	6.2 - 95.6	49.4	67	0.980	0.980
Folpan® 80 WDG	10	50.0	25.4 - 65.0	6.9 - 89.3	47.8	62	0.782	0.782
Funguran® progress	100	59.5	15.9 - 78.1	6.6 - 95.3	50.9	65	0.900	0.980
SpinTor™	100	44.9	17.8 - 71.5	7.7 - 96.1	45.7	58	0.632	0.885
Taifun® forte	100	22.3	18.3 - 61.2	11.7 - 75.0	35.9	22	**0.016**	**0.036**
Taifun® forte	10	45.5	31.5 - 58.4	9.6 - 85.2	46.9	57	0.597	0.782
Vivando®	100	25.9	17.1 - 40.2	2.8 - 81.4	31.2	17	**0.006**	**0.022**
Vivando®	10	58.4	28.9 - 86.8	10.8 - 94.7	55.9	86	0.375	0.751
Wettable Sulphur Stulln	100	33.0	20.1 - 46.3	6.4 - 83.5	37.0	28	**0.039**	0.068

The reduction of the sampling interval did not result in significant differences in the proportion of time spent on the contaminated side (all p > 0.144 when testing t_{pest} against t_{pest_10}, t_{pest_60}, t_{pest_900} or t_{pest_3600}; Fig 2 and Table 3), with the exception of t_{pest_60} in Taifun® forte. Also the overall trend to prefer one side stayed the same when comparing t_{pest_10}, t_{pest_60}, t_{pest_900} or t_{pest_3600} against a random side choice (50%, Table 3). However, without adjusting the p-values with the FDR, significance was lost for Wettable Sulphur Stulln at a sample interval of one sample every 15 minutes (t_{pest_900}), and for Taifun® forte at a sample interval of one sample every hour (t_{pest_3600}) when using the FDR (Table 3). Restricting the study time to the first hour of the test (t_{pest_1h}) resulted in significant differences to t_{pest} in Folpan® 500 SC and Vivando® (Fig 2 and Table 3). When testing t_{pest_1h} against a random side choice no significant avoidance of the contaminated side was found for any tested formulation.

In the three choice-tests in which we observed a significant difference between t_{pest} and a random side choice, also significant differences in the activity of the toads were found (Table 4). The median distance a toad moved on the contaminated side per hour was on average 5.1 times longer for Folpan® 500 SC, 2.3 times longer for Vivando®, and 2.5 times longer for Taifun® forte than the distance moved on the uncontaminated side. In all other choice-tests no activity differences were observed (Fig 3 and Table 4).

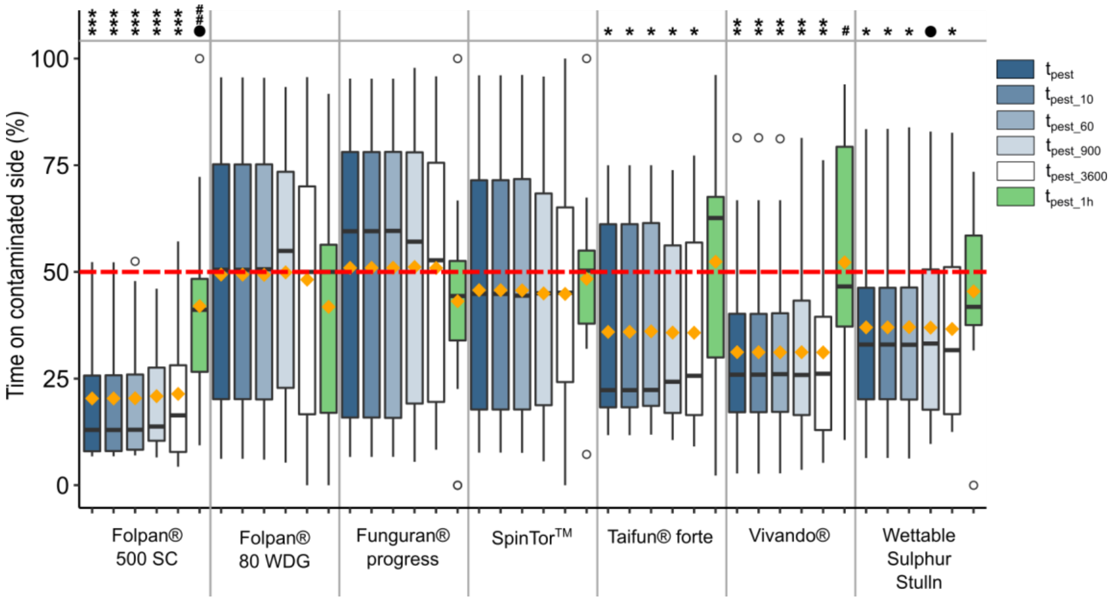

Fig. 2: Boxplots showing the proportion of time a toad spent on the contaminated side of an arena for each tested formulation at the maximum recommended field rate (FR_{max}) and for different sampling intervals. For the calculation of tpest all data over 24 hours were used. For t_{pest_10}, t_{pest_60}, t_{pest_900} and t_{pest_3600} only one side choice every 10, 60, 900 and 3,600 seconds, respectively, were considered. t_{pest_1h} contains only data from the first hour of an experimental run. In each boxplot, the boundaries of the box are the 25th and 75th percentiles and the whiskers correspondent to the lowest and largest value no further than 1.5 times from the 25th and 75th percentiles away. Data points beyond the whiskers are shown as unfilled circles. Median values are presented as horizontal lines and orange diamonds show the mean values. Significant difference from a random choice (50%; red dotted line): ●: p adj. < 0.1; *: p adj. < 0.05; **: p adj. < 0.01; ***: p adj. < 0.001. P-values from tests with the same sampling interval were adjusted using the FDR. Significant differences compared to tpest: # = p < 0.05; ### = p < 0.001. N = 16 per choice-test.

Table 3: Proportion of time a toad spent on the contaminated side of an arena for each tested formulation at the maximum recommended field rate (FR_{max}) and for different sampling intervals.

Formulation	Sampling interval	Time on contaminated side (%) t_{pest}				Wilcoxon-Test - comp. to 50%			Wilcoxon-Test - comp. to t_{pest}	
		Median	IQR	Range	Mean	V	p	p adj.	V	p
Folpan® 500 SC	t_{pest}	13.0	8.0 - 25.7	6.8 - 52.3	20.3	2	**< 0.001**	**< 0.001**	not tested	
	t_{pest_10}	13.0	8.0 - 25.8	6.8 - 52.2	20.3	2	**< 0.001**	**< 0.001**	65	0.900
	t_{pest_60}	13.0	8.3 - 26.0	7.0 - 52.5	20.4	2	**< 0.001**	**< 0.001**	56	0.562
	t_{pest_900}	13.8	10.4 - 27.6	6.5 - 46.1	20.9	0	**< 0.001**	**< 0.001**	52	0.433
	t_{pest_3600}	16.4	7.8 - 28.1	4.3 - 57.1	21.4	3	**< 0.001**	**0.006**	54	0.495
	t_{pest_1h}	41.1	26.6 - 48.4	9.4 - 100.0	42.0	34	0.083	0.583	7	**< 0.001**
Folpan® 80 WDG	t_{pest}	50.5	20.2 - 75.2	6.2 - 95.6	49.4	67	0.980	0.980	not tested	
	t_{pest_10}	50.5	20.2 - 75.2	6.2 - 95.6	49.4	67	0.989	0.980	94	0.193
	t_{pest_60}	50.6	20.1 - 75.2	6.0 - 95.5	49.3	67	0.980	0.980	80	0.562
	t_{pest_900}	54.9	22.8 - 73.5	5.3 - 93.3	49.9	69	0.980	1.000	54	0.495
	t_{pest_3600}	50.0	16.6 - 70.0	0.0 - 95.7	48.2	47	0.754	0.879	84	0.433
	t_{pest_1h}	50.0	17.0 - 56.4	0.0 - 91.7	41.8	46	0.454	0.835	78	0.330
Funguran® progress	t_{pest}	59.5	15.9 - 78.1	6.6 - 95.3	50.9	65	0.900	0.980	not tested	
	t_{pest_10}	59.6	15.9 - 78.1	6.6 - 95.3	51.0	65	0.900	0.980	42	0.193
	t_{pest_60}	59.6	15.8 - 78.1	6.7 - 95.3	51.0	65	0.980	0.980	44	0.231
	t_{pest_900}	57.1	19.2 - 78.0	5.5 - 97.8	51.1	68	1.000	1.000	63	0.821
	t_{pest_3600}	52.7	19.5 - 75.6	8.3 - 95.8	50.9	69	0.980	0.979	73	0.821
	t_{pest_1h}	44.4	33.9 - 52.6	0.0 - 100.0	43.1	41	0.170	0.596	99	0.117
SpinTor™	t_{pest}	44.9	17.8 - 71.5	7.7 - 96.1	45.7	58	0.632	0.885	not tested	
	t_{pest_10}	44.9	17.8 - 71.5	7.7 - 96.1	45.7	58	0.632	0.885	72	0.860
	t_{pest_60}	44.4	17.8 - 71.7	7.6 - 96.2	45.7	56	0.562	0.789	85	0.404
	t_{pest_900}	45.0	18.8 - 68.4	5.6 - 95.8	45.0	56.5	0.570	0.797	90	0.274
	t_{pest_3600}	45.1	24.2 - 65.1	0.0 - 100.0	44.8	53.5	0.469	0.657	82	0.495
	t_{pest_1h}	50.3	37.9 - 55.0	7.2 - 100.0	48.4	57	0.597	0.835	61	0.744
Taifun® forte	t_{pest}	22.3	18.3 - 61.2	11.7 - 75.0	35.9	22	**0.016**	**0.036**	not tested	
	t_{pest_10}	22.3	18.3 - 61.2	11.7 - 75.0	36.0	22	**0.016**	**0.036**	39	0.144
	t_{pest_60}	22.3	18.6 - 61.5	11.9 - 75.0	36.1	22	**0.016**	**0.036**	23	**0.018**
	t_{pest_900}	24.3	17.0 - 56.2	10.6 - 73.9	35.8	24	**0.021**	**0.050**	68	1.000
	t_{pest_3600}	25.7	16.5 - 56.9	9.1 - 77.3	35.7	25.5	**0.030**	0.067	71	0.900
	t_{pest_1h}	62.6	30.0 - 67.6	2.3 - 96.1	52.4	71	0.900	0.900	35	0.093
Vivando®	t_{pest}	25.9	17.1 - 40.2	2.8 - 81.4	31.2	17	**0.006**	**0.022**	not tested	
	t_{pest_10}	25.9	17.1 - 40.2	2.7 - 81.4	31.2	17	**0.006**	**0.022**	87	0.348
	t_{pest_60}	26.0	17.2 - 40.3	2.8 - 81.2	31.2	17	**0.006**	**0.022**	71	0.900
	t_{pest_900}	25.9	16.5 - 43.3	3.6 - 81.4	31.2	19	**0.009**	**0.032**	68	1.00
	t_{pest_3600}	26.1	12.9 - 39.5	5.3 - 76.2	31.1	17	**0.006**	**0.022**	53	0.464
	t_{pest_1h}	46.6	37.2 - 79.3	10.6 - 94.0	52.2	49	0.839	0.900	15	**0.033**
Wettable Sulphur Stulln	t_{pest}	33.0	20.1 - 46.3	6.4 - 83.5	37.0	28	**0.039**	0.068	not tested	
	t_{pest_10}	33.0	20.2 - 46.3	6.4 - 83.5	37.0	28	**0.039**	0.068	53	0.464
	t_{pest_60}	32.9	20.1 - 46.3	6.3 - 83.9	37.1	29	**0.044**	0.078	53	0.464
	t_{pest_900}	32.5	17.7 - 50.6	9.7 - 82.9	36.9	27	0.065	0.114	78	0.632
	t_{pest_3600}	31.7	16.7 - 51.5	12.5 - 82.6	36.6	23	**0.038**	0.067	77	0.669
	t_{pest_1h}	41.8	37.5 - 58.5	0.0 - 73.5	45.4	48	0.524	0.835	38	0.229

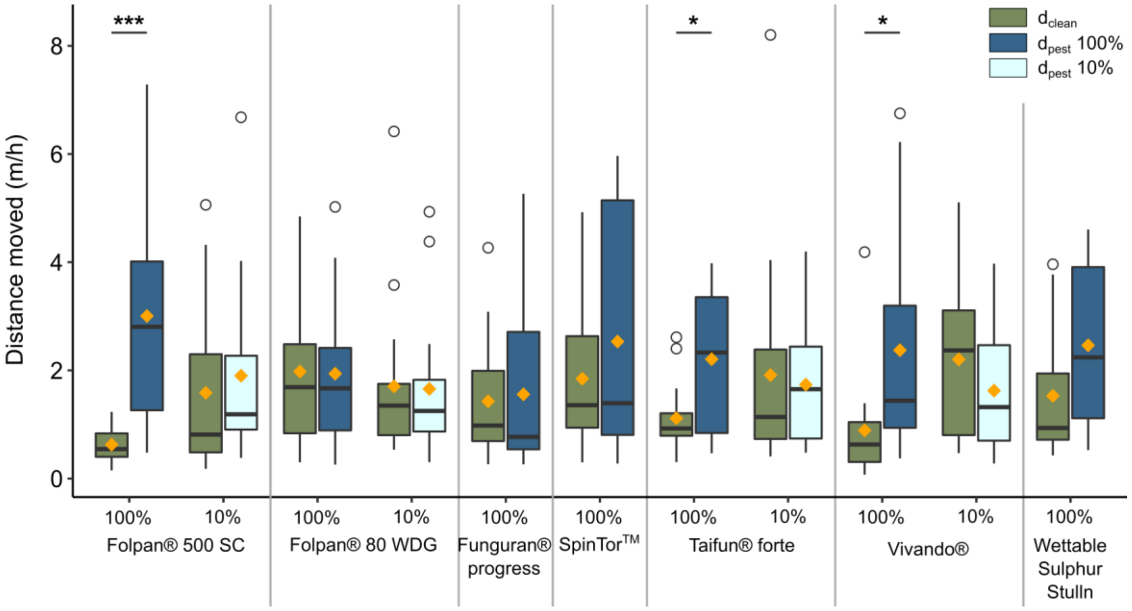

Fig. 3: Boxplots showing the distance moved in meter per hour on the contaminated (d_{pest}; dark blue = 100% of the maximum recommended field rate (FR_{max}), light blue = 10% of FR_{max}) and uncontaminated side (d_{clean}; green) of an arena over 24 hours. In each boxplot, the boundaries of the box are the 25th and 75th percentiles and the whiskers correspondent to the lowest and largest value no further than 1.5 times from the 25th and 75th percentiles away. Data points beyond the whiskers are shown as unfilled circles. Median values are presented as horizontal lines and orange diamonds show the mean values. Significant difference between d_{pest} and d_{clean}: *: p adj. < 0.05; ***: p adj. < 0.001. P-values from tests with the same concentration were adjusted using the FDR. N = 16 per choice-test.

Table 4: Distances moved in meter per hour on the contaminated (pest; 10% or 100% of the of the maximum recommended field rate (FR_{max}); d_{pest}) and uncontaminated (clean; d_{clean}) side of an arena and results from two-sided Wilcoxon signed-rank tests that were used to compare d_{pest} and d_{clean}.

Formulation	% of FR_{max}	Side	Distance moved (m/h)				Wilcoxon-Test - clean vs. pest		
			Median	**IQR**	**Range**	**Mean**	**V**	**p**	**p adj.**
Folpan® 500 SC	100	Clean	0.55	0.40 - 0.84	0.15 - 1.23	0.63	0	< 0.001	< 0.001
		Pest	2.81	1.26 - 4.01	0.48 - 7.28	3.01			
Folpan® 500 SC	10	Clean	0.81	0.49 - 2.30	0.18 - 5.06	1.58	58	0.632	0.701
		Pest	1.19	0.91 - 2.27	0.38 - 6.68	1.90			
Folpan® 80 WDG	100	Clean	1.69	0.84 - 2.48	0.30 - 4.85	198	72	0.860	0.860
		Pest	1.67	0.89 - 2.42	0.26 - 5.02	1.94			
Folpan® 80 WDG	10	Clean	1.35	0.80 - 1.75	0.53 - 6.42	1.70	70	0.934	0.782
		Pest	1.25	0.87 - 1.83	0.30 - 4.93	1.66			
Funguran® progress	100	Clean	0.98	0.69 - 1.99	0.27 - 4.27	1.43	64	0.860	0.860
		Pest	0.77	0.54 - 2.71	0.26 - 5.26	1.56			
SpinTor™	100	Clean	1.36	0.94 - 2.63	0.30 - 4.92	1.85	52	0.433	0.606
		Pest	1.39	0.81 - 5.14	0.28 - 5.97	2.53			
Taifun® forte	100	Clean	0.93	0.79 - 1.21	0.30 - 2.61	1.11	24	0.021	0.050
		Pest	2.33	0.84 - 3.35	0.47 - 3.98	2.21			
Taifun® forte	10	Clean	1.14	0.73 - 2.39	0.41 - 8.20	1.91	66	0.934	0.782
		Pest	1.65	0.74 - 2.44	0.48 - 4.20	1.74			
Vivando®	100	Clean	0.63	0.31 - 1.04	0.07 - 4.19	0.89	17	0.006	0.022
		Pest	1.44	0.94 - 3.20	0.37 - 6.75	2.37			
Vivando®	10	Clean	2.37	0.80 - 3.11	0.47 - 5.11	2.20	86	0.376	0.751
		Pest	1.32	0.70 - 2.47	0.28 - 3.97	1.63			
Wettable Sulphur Stulln	100	Clean	0.94	0.72 - 1.94	0.43 - 3.96	1.53	31	0.058	0.101
		Pest	2.24	1.12 - 3.91	0.53 - 4.61	2.46			

Discussion

Based on over 2,300 hours of video recordings, we found evidence of an avoidance behavior of common toad juveniles for three out of seven tested pesticide formulations at maximum recommended field rates. For one other formulation a trend for avoidance could be observed. As we could exclude the presence of external cues or a cardinal direction with the control-test, the observed side choice can be traced back to the pesticide. Overspraying the surface with the maximum recommended field rate represents a worst-case scenario. Fungicides and insecticides are usually applied directly on the plant, resulting in an interception by the crop and therefore a reduced concentration on the ground [50]. However, especially fungicides are applied several times per year with short time periods between applications and often as mixtures of several formulations [8, 46, 51], increasing the overall soil pesticide load. Further, herbicides like the tested glyphosate formulation Taifun® forte are usually directly applied on the ground. Therefore, contamination of the soil with the field rate is a worst-case, but still realistic scenario.

To avoid a contaminated surface, toads have to be able to detect the contamination. As the used formulations did not dye the silica sand, visual detection is unlikely. Therefore, the detection is likely to be related to olfactory or somatosensory perception, or internal mechanisms like a metabolic response that triggers a purpose-orientated behavior and presupposes the uptake of the substance. As amphibians have a highly permeable skin [23], an uptake is possible when they come in contact with contaminated soil [11, 12]. However, as shown for the common wall lizard (*Podarcis muralis*) [52], the metabolic response might be time-delayed, making it unlikely for the toad to link the metabolic response to the pesticide exposure and to subsequently react with an avoidance of a contaminated surface. In Storrs Méndez et al. [11] an uptake of atrazine was demonstrated for the American toad (*B. americanus*), but even after 60 hours, no avoidance behavior was observed. Amphibians have a good olfactory perception and use chemical cues for example during courtship [21] or for orientation [22]. Juvenile *B. bufo* are able to perceive and recognize olfactory cues from different sources, e.g. lake water [53]. Farabaugh and Nowakowski [54] demonstrated that the strawberry poison frog (*Oophaga pumilio*) can use olfactory cues to detect the glyphosate herbicide Roundup™. Therefore, the detection of olfactory cues from contaminated surfaces might be possible. However, it remains unknown if the differentiation of contaminated and uncontaminated areas based on olfactory cues is possible in an arena with a diameter of only 20 cm like in our setup. Compared to the olfactory perception, the somatosensory perception might be more independent from the dimensions of the arena and the contaminated and uncontaminated areas. The active ingredient or at least one of the additives of all three avoided pesticide formulations, as well as of Wettable Sulphur Stulln, where a trend to avoidance could be found, are classified as "Causes severe skin burns and eye damage" or "Causes skin irritation". This is not the case for the other tested formulations, even though Folpan® 80 WDG is classified as "May cause an allergic skin reaction" (Tab. 1). Therefore, these classifications could be an indicator for an avoidance behavior. However, some classified additives can only be found in small amounts in the formulation (e.g. < 0.1% 3-Benzisothiazolinon in Folpan® 500 SC) and also the number of tested formulations is too low to draw any general conclusion. Therefore, the physiological mechanisms of the avoidance remain unknown, and could also be different between formulations.

Interestingly, we found a significant avoidance of Folpan® 500 SC, but not of Folpan® 80 WDG. Both formulations have the same active ingredient folpet and were tested in their maximum recommended field rate, which results in a comparable amount of the active ingredient (1.20 and 1.28 kg a.i/ha). Therefore, toads might not be able to detect folpet. Observed differences in the avoidance cannot be explained by the active ingredient, but might be the result of additives in the formulation. Additives change the characteristics of the formulation and several studies showed that they can enhance or decrease toxic effects [17, 55, 56]. Folpet is classified as "May cause an allergic skin reaction", but 3-benzisothiazolinone, an additive only in Folpan® 500 SC, is also classified as "Causes skin irritation", which might affect the avoidance behavior. Individuals tested on Folpan® 500 SC were captured in the beginning of August, while individuals used for Folpan ® 80 WDG were captured in the beginning of September, so were about one month older and also differed in their body weight (S1 Table). It cannot be ruled out that these differences influenced the behavior during the tests and therefore caused the contrasting results among the two folpet formulations. Due to the variability between experimental runs in weight/size and age of the individuals, but also in the time the toads were kept in the cages before the experiment or the exact starting time of the experiment (S1 Table), comparisons among experimental runs can only be made with caution. Differences in the age, but also differences in the habitat use (i.e. the time spent in vineyards) might also come with differences in the exposure to pesticides before the experimental run. As each pesticide was tested only once at 10 or 100% of FR_{max}, general conclusion if and how all these factors affect the avoidance behavior cannot be stated. Thus, their combined effects should be examined in future studies in detail.

In previous studies, amphibians were able to detect and therefore avoid pesticides in the laboratory on artificial surfaces like filter paper, but usually not on more natural soils. Hatch et al. [29] conducted choice tests with urea, which is used as fertilizer in agriculture and forestry. Juvenile western toads (*Bufo boreas*) and cascades frogs (*Rana cascadae*) avoided urea-dosed paper towels in an arena experiment, but showed no preference when a natural substrate was used. In contrast, Gaglione et al. [30] found avoidance of urea both on contaminated filter paper as well as commercial top soil for the red-backed salamander (*Plethodon cinereus*). Gertzog et al. [31] showed that *P. cinereus* also avoids filter paper contaminated with three different herbicide formulations. Also Iberian newts (*Lissotriton boscai*, formerly *Triturus boscai*) avoid filter paper dosed with the fertilizer ammonium nitrate [32]. Storrs Méndez et al. [11] conducted choice tests with the herbicide atrazine on soil. Although atrazine was absorbed by juvenile American toads (*Bufo americanus*), no avoidance could be detected. In terms of environmental realism, we rank the silica sand used in our study system as intermediate between studies with contaminated filter paper and natural soil. Although loamy to sandy soils can be found in vineyards, organic components are completely lacking in the sand we used, which is unrealistic for natural soils. The organic matter content of soils affects the bioavailability, uptake and thus bioaccumulation of pesticides by amphibians [57], and could therefore also play a role in the avoidance behavior. We chose the silica sand mainly because of its coloration, as its brightness increased the contrast to the dark toads. Most natural soils would have been darker, thus decreasing the contrast to the experimental animal and increasing the probability of errors during the automatic detection of the toads in the arenas by EthoVision® XT. Natural soils could be tested when side choice is determined manually without a tracking software. However, this would require the reduction of the sampling interval. A reduction to every 3,600 seconds (= 1 hour; resulting in 24 frames when filming for 24 hours) or 900 seconds (= 15 minutes; 360 frames over 24 hours) would allow determining the side choice manually without a tracking software. The reduction to one data point every 10 or 60 seconds would only allow to speed up the, in some cases long-lasting, analysis with the tracking software. In general, the reduction can be expected to have only little effect on the proportion of time spent on a side, as differences presuppose that toads are very active and are changing the side frequently. However, in cases where the avoidance behavior is only weak, also small differences might result in an increased probability of false-positive or false-negative results. In our study, a weak avoidance behavior was observed for Taifun® forte at a sample interval of one sample per hour (t_{pest_3600}; p = 0.030). Nevertheless, in a screening of several pesticide formulations, one has to consider the probability of a type I error, and thus adjust the p-values of statistical tests, which resulted in the loss of significance in Taifun® forte (t_{pest_3600}). P-value adjustment also resulted in p-values above the criterion of significance (0.05) for t_{pest} and all subsamplings of t_{pest} when testing Wettable Sulphur Stulln. Thus, the same avoidance response of the toads to the pesticide was found for all sample intervals. However, when solely regarding Wettable Sulphur Stulln without using the FDR, a significant avoidance behavior was found for t_{pest}, t_{pest_10}, t_{pest_60}, and t_{pest_3600}, but not for t_{pest_900}. Thus, both a sampling interval of one sample per 15 min and one sample per hour could have led to an overlooked avoidance behavior in one pesticide formulation. When the data was limited to the first hour of an experiment, no avoidance behavior could be detected for any tested pesticide. Some toads did not move at all during the first hour, underlining the importance of a prolonged acclimatization period. Future studies on amphibian avoidance behavior should be aware of these problems and should not neglect cases where no significance, but a trend is found, e.g. when it comes to choosing formulations for a higher-tier-assessment. As we found high variability in the behavior of tested toads, we would further recommend to increase the number of replicates, if possible.

Alterations of the movement behavior after pesticide exposure are well known for amphibians. An abnormal swimming behavior and a decreased activity of larvae can often be observed [37, 58], whereby such alterations are usually induced by the neurotoxicity of the pesticide [59]. In our study, differences in the distance moved per hour on the contaminated *versus* the uncontaminated side might be rather linked to the avoidance behavior, in the sense that toads might have avoided resting on the contaminated side for longer periods. Consequently, we found increases in the moved distance on the contaminated side in the choice-tests with Folpan® 500 SC, Vivando® and Taifun® forte. In general, most studies on amphibian behavioral response to pesticides are focusing on the larval stages in an aquatic environment [37], which corresponds to the underrepresentation of terrestrial life stages in ecotoxicological studies [60]. Considering the high toxicity of some pesticides for terrestrial amphibians [16–18], the numerous studies on effects in the aquatic habitat [37] and the effects of pesticides on the behavior of other ectothermic groups like lizards [61], it is likely that pesticides can also alter the behavior of terrestrial amphibians. However, most studies on the effects of pesticides on terrestrial amphibians did not find evidence for behavior alterations (see review in [60]). One explanation might be a lack of standardized methods and adequate endpoints to study these alterations. To our surprise, we found no ecotoxicological study in which automatic video tracking of exposed individuals was used in terrestrial amphibians, although this method is often used in a variety of taxa like bees [62], green lacewings [63] or mice [64] and also for aquatic amphibian larvae [65, 66]. This method might provide informative endpoints in future terrestrial amphibian studies in an upcoming pesticide risk assessment for amphibians. The setup we used, which is based on a Raspberry Pi, might help researchers to study these aspects, as it allows the filming of multiple individuals in parallel and it is a simple, freely configurable and affordable alternative to specialized video equipment. Besides highly professional tracking software like EthoVision there is also a rising number of open-source, freely available alternatives [67].

We detected avoidance of three out of seven tested pesticide formulations at 100% of $FR_{max,}$ and no avoidance when using a concentration of 10% of FR_{max} in any formulation. As agriculture with frequent pesticide applications is the dominant type of land use in many regions, an avoidance might contribute to a chemical landscape fragmentation. Landscape fragmentation can lead to reduced gene flow between, and as a result, reduced fitness of amphibian populations [68]. On the other hand, the lack of avoidance behavior in the other tested formulations might increase the pesticide exposure risk of amphibians in agricultural landscapes, which could lead to sublethal [13–15] and lethal effects, even at field rates of 10% [17]. Therefore, we conclude that a heterogeneous landscape with green corridors between populations and different habitat types is needed so that contaminated areas can be avoided without leading to a fragmentation of the landscape. Future studies on behavior choice tests should consider adult individuals, natural soils with different contents of organic matter as well as soils that have been oversprayed not directly before the test allowing adsorption to soil to represent other potential scenarios. Testing individuals from uncontaminated populations would help to understand whether the avoidance is an evolved adaption. Future tests should also reflect realistic application sequences with mixtures of multiple pesticides [69]. Last but not least, field studies are needed to verify results from laboratory studies under realistic conditions.

Additional Information and Declarations

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Data Availability

All relevant data are within the manuscript, its Supporting Information files or are available from the data repository Zenodo (DOI: 10.5281/zenodo.3989024).

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Competing interests

The authors have declared that no competing interests exist.

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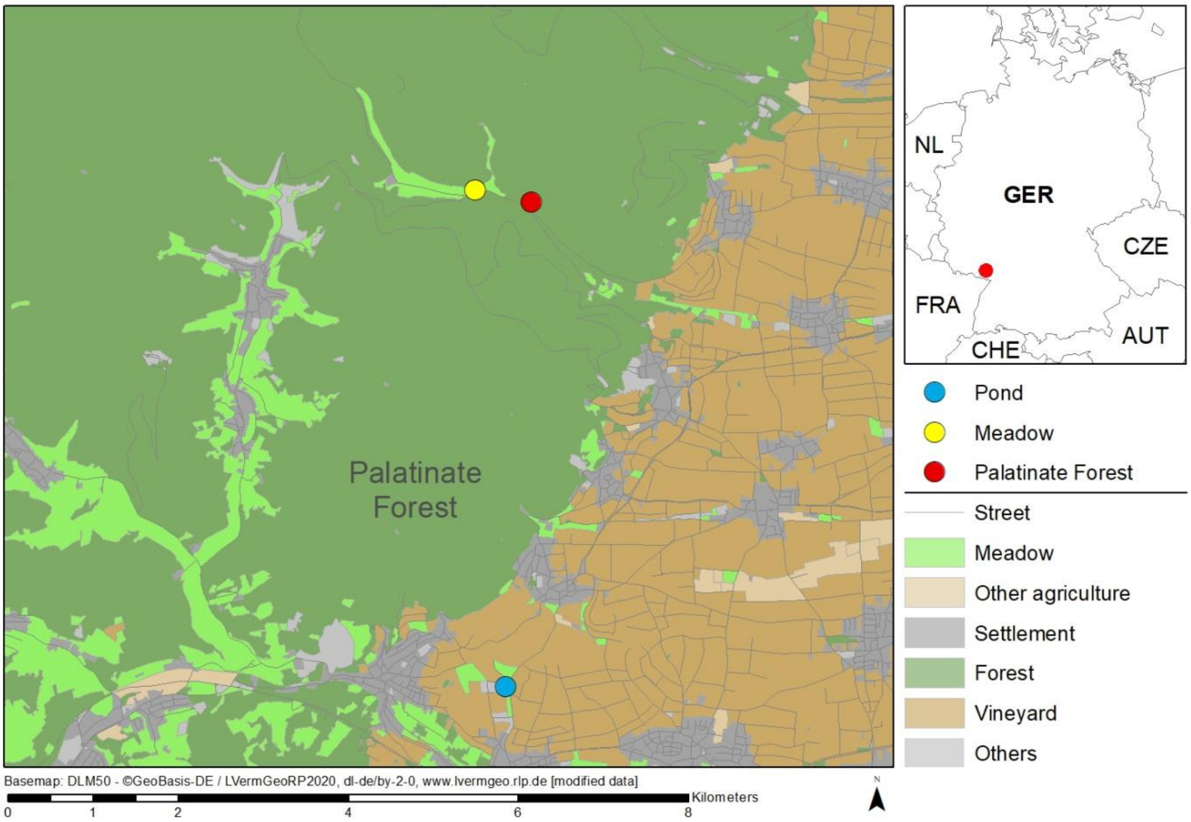
Supporting information

S1 Table: Detailed information about each choice test. The table includes the date when the toads were captured, the date of the experimental run, the times when the first and the last toad were placed in the test arenas, the mean weight with its standard deviation (SD) of the toads used in each test as well the proportion of the total time a toad spent in a 2.5 cm wide area at the border between the contaminated and uncontaminated side of an area (buffer zone). Positions of toads in the buffer zone were excluded when analyzing the avoidance behavior. FR$_{max}$ is the maximum recommended field rate of a formulation.

Formulation	% of FR$_{max}$	Date of toad sampling	Date of test	Start time first toad	Start time last toad	Weight (mg)		Time in buffer zone (%)			
						Mean	SD	Median	IQR	Range	Mean
Folpan® 500 SC	100	01.08.2018	14.08.2018	12:17	12:34	293.1	42.6	6.1	2.7 - 15.9	1.5 - 46.6	12.7
Folpan® 500 SC	10	24.08.2018	30.08.2018	11:16	11:33	372.7	48.7	5.8	4.3 - 11.5	0.8 - 52.9	12.5
Folpan® 80 WDG	100	03.09.2018	13.09.2018	11:14	11:30	433.7	115.5	12.2	6.4 - 24.8	2.0 - 43.3	17.1
Folpan® 80 WDG	10	01.08.2018	07.08.2018	12:27	12:42	368.6	52.3	15.8	10.2 - 34.6	2.0 - 45.2	20.0
Funguran® progress	100	09.08.2018	16.08.2018	12:26	12:44	306.6	49.8	10.2	4.5 - 16.2	3.2 - 36.2	12.2
SpinTor™	100	29.08.2018	11.09.2018	11:45	12:04	442.1	84.9	13.9	6.1 - 21.2	1.5 - 40.4	15.1
Taifun® forte	100	25.07.2018	09.08.2018	13:29	13:45	333.8	71.7	10.5	6.7 - 15.4	3.8 - 60.2	15.0
Taifun® forte	10	13.08.2018	28.08.2018	10:58	11:20	362.4	58.6	22.4	11.0 - 31.6	5.3 - 59.3	24.2
Vivando®	100	09.08.2018	21.08.2018	12:27	12:43	305.5	60.8	8.2	3.0 - 17.5	1.6 - 54.9	14.9
Vivando®	10	24.08.2018	04.09.2018	11:07	11:20	384.6	75.0	12.1	4.8 - 35.5	2.1 - 58.5	20.6
Wettable Sulphur Stulln	100	19.09.2018	20.09.2018	12:07	12:24	552.6	88.6	7.8	3.9 - 18.6	2.7 - 51.6	13.7

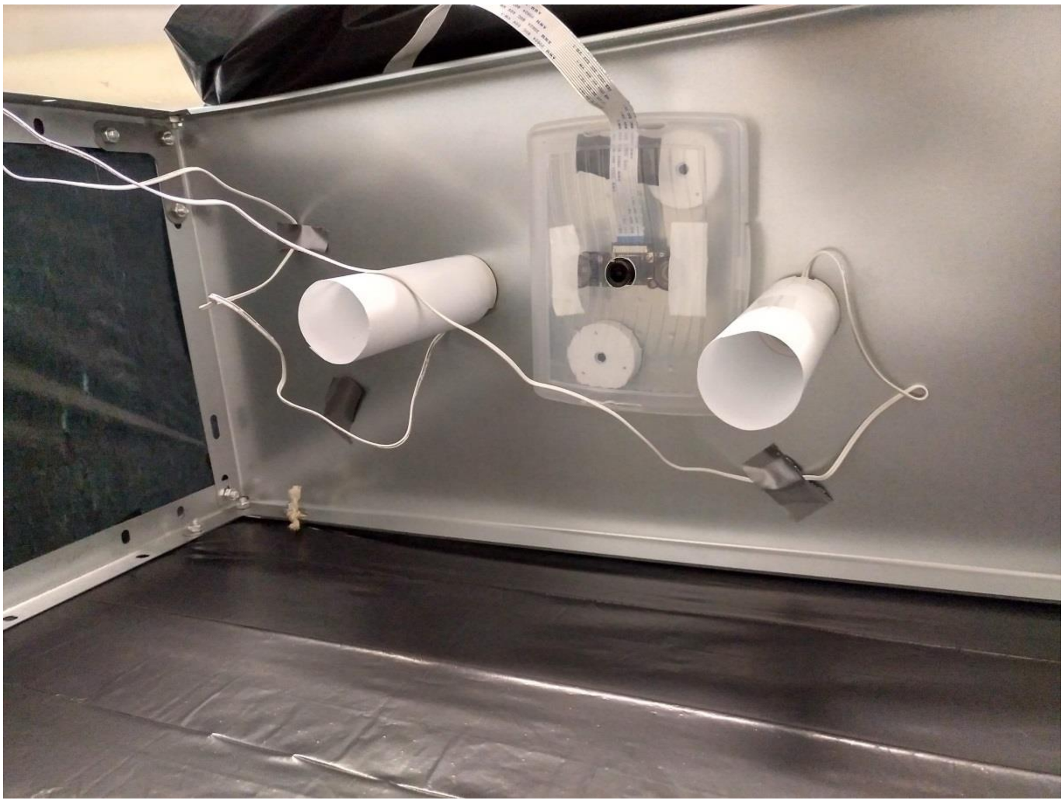


Basemap: DLM50 - ©GeoBasis-DE / LVermGeoRP2020, dl-de/by-2-0, www.lvermgeo.rlp.de [modified data]

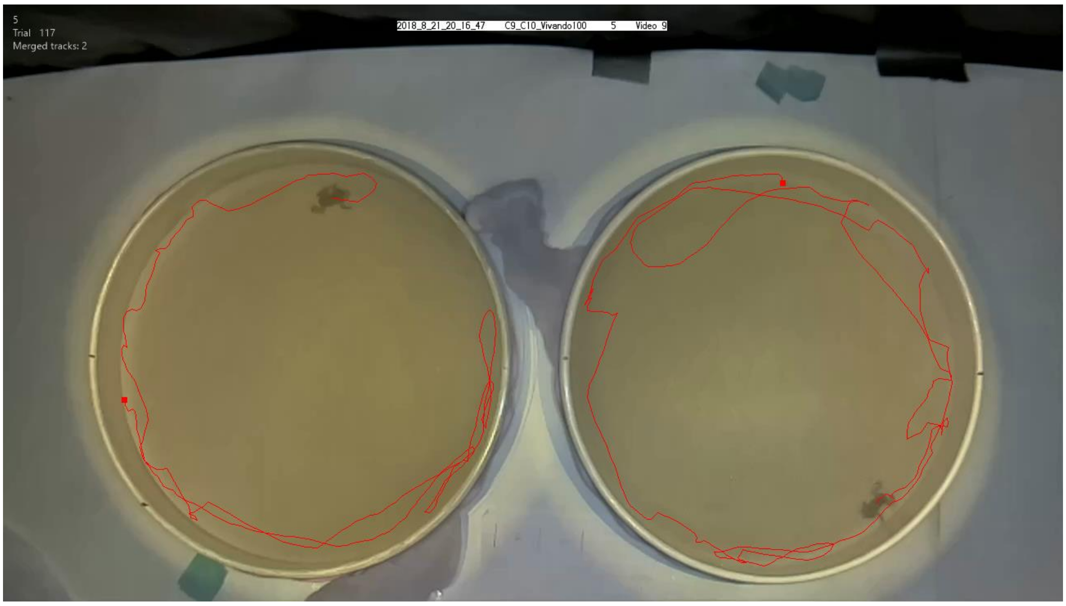
S1 Fig.: Map of the study area. The points show the location of the pond where the individuals for the experimental runs were captured (blue; "Pond"), the location where insects for feeding of the toads were captured (yellow; "Meadow") and the location where soil, moss and leaves were collected to equip the outdoor net cages (red; "Palatinate Forest"). Reprinted from www.lvermgeo.rlp.de under a CC BY license, with permission from GeoBasis-DE / LVermGeoRP2020, original copyright 2020.

S2 Fig.: Glass petri dish filled with silica sand before the pesticide application. One side is covered with laminated paper semicircles to prevent contamination of the clean side during the application process.

S3 Fig.: Two test arenas with experimental animals in a test chamber right before test start. Arenas are covered with a polyamide fabric.



S4 Fig.: Photo of the camera module (SC15; Kuman Ltd., Shenzhen, China) that was used to record the toads and used LED lights. The camera was attached to a Raspberry Pi (Raspberry Pi 3 Model B; Raspberry Pi Foundation, Cambridge, UK).



S5 Fig.: Screenshot of a video recorded during one of the choice tests showing experimental animals in their arena with the visualization of the track of the animals from EthoVision® XT.

Chapter 3

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

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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.

Keywords

Landscape genetics, Microsatellites, Amphibians, Common frog, Isolation by distance, Agriculture

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, Runborg & Sjögren-Gulve, 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, Pfeffer & Kalettka, 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; Lenhardt, Brühl & Berger, 2014; Joseph, 2016).

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, Brühl & Berger, 2014). 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 (Kumar, 2014; Brodeur et al., 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 (Sparling & Fellers, 2009; Relyea, 2011; Denoël et al., 2013; Ghose et al., 2014; Lau, Karraker & Leung, 2015) 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 & Arntzen, 2002). By combining several microsatellite markers it is possible to estimate genetic differentiation among adjacent populations (Beebee & 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.

Material and Methods

The study was conducted in Rhineland-Palatinate, Germany, between Neustadt/Weinstrasse and Landau/Pfalz (Fig. 1; Figs. S1–S3). 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. 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 (Figs. S4). 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.

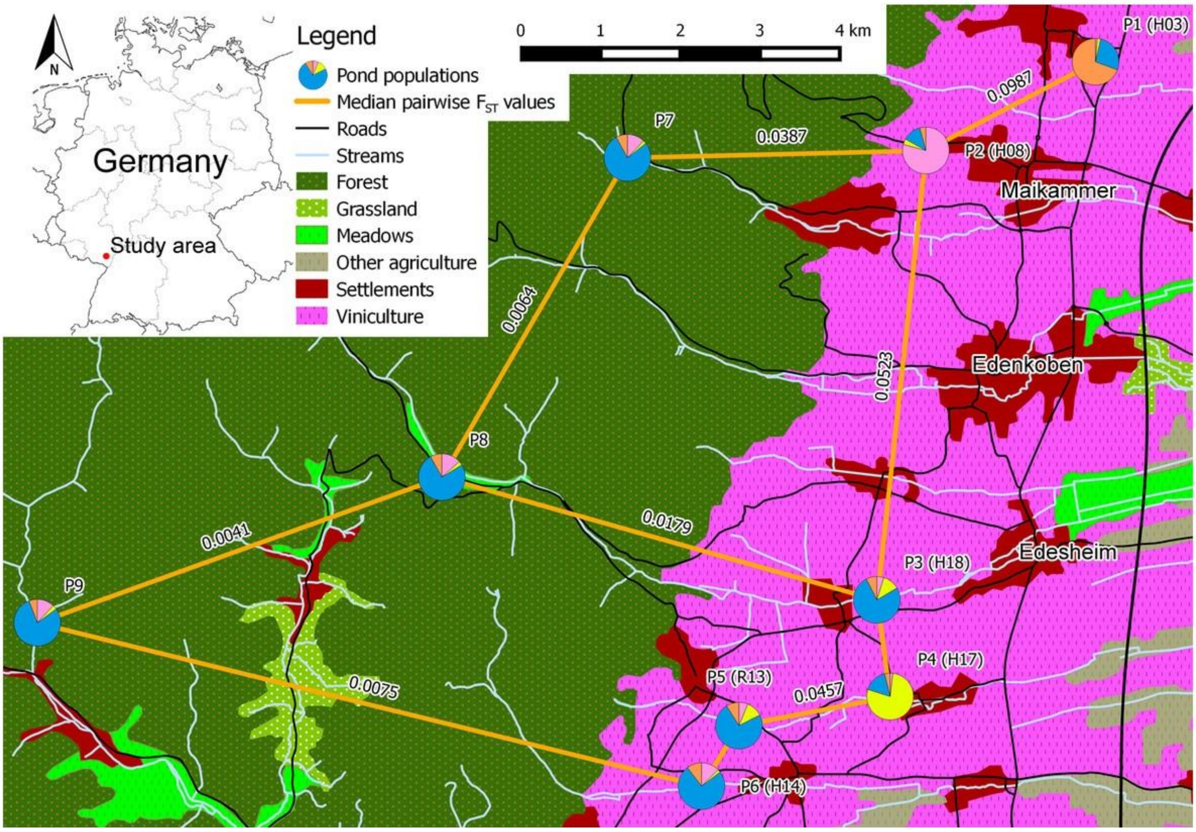

Fig. 1: Schematic overview of the core study area in southern palatinate between “Neustadt an der Weinstraße” (north of P1) and “Landau in der Pfalz” (south of P6) with median pairwise FST values for selected pond population pairs. Pond labels of Lenhardt et al. (2013) in brackets. Pie charts of the 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).

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, Lenhardt & Theissinger, 2013). We applied a high salt DNA extraction protocol to obtain DNA from tissue samples of the tadpoles (Aljanabi & Martinez, 1997).

We analyzed ten variable microsatellite loci (Table 1; Matsuba & Merilä, 2009) and amplified the fragments in two multiplex PCRs using the QIAGEN Multiplex PCR Kit (Hilden, Germany) following Müller, Lenhardt & Theissinger (2013). The selected loci were chosen from a number of tested loci due to their amplification success and polymorphism in an earlier study (Müller, Lenhardt & Theissinger, 2013). Also, six of the selected loci were located on different chromosomes (Table 1; 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).

Table 1: 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 with an asterisk (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

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 & 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 & 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, 2002).

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 & 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, Stephens & Donnelly, 2000) was used. Since the presence of siblings can also bias the detection of genetic clusters (Anderson & Dunham, 2008; Rodriguez-Ramilo & 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, Wen & Falush, 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 & VonHoldt, 2012) was used. Results were combined with the LargeKGreedy algorithm with 10, 000 random input orders in CLUMPP (Jakobsson & 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; Tables S1 and S2) 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, Pfeffer & Kalettka, 2011), 200 m wide strips take possible deviations from this average daily migration distance, resulting for example from foraging, into account (see also Vos et al., 2001; Arens et al., 2007).

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 (Vos et al., 2001; Arens et al., 2007), 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 2), since each habitat type may impact the genetic differentiation with a different magnitude. 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 Tables 2 and 3) 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 2: Overview of all weighted distance models.

Weighted distance models	Description
LGD*RNA	Linear geographic distance (LGD) weighted for the fraction of negative area (NA). RNA 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) RNA = (NA∗HCF + TA)⁄TA
LGD*RPA	Linear geographic distance (LGD) weighted for the fraction of positive area (PA). RPA 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) RPA = TA⁄(PA∗HCF + TA)
LGD*RNA*RPA	Combined weighted distance for positive and negative area.

Table 3: Results of isolation by distance for median pairwise FST (MPF) as well as median pairwise RST (MPR) and the linear geographic distance (LGD) corrected by the weighted distance models with habitat correction factor (HCF).

Weighted distance model		MPFs			MPRs	
	HCF	***p*-value**	R^2	**HCF**	***p*-value**	R^2
LGD*RNA viniculture	10. 8	<0.001	0.327	07. 3	0.008	0.159
LGD*RNA settlements	88.5	0.125	0.107	1.0	0.153	0.040
LGD*RPA forest	08. 8	0.005	0.303	4.0	0.016	0.079
LGD*RPA grassland	16. 2	0.365	0.043	38.5	0.239	0.069
LGD*RPA meadows	11.6	0.165	0.302	10. 3	0.092	0.140
LGD*RPA copse	1.0	0.288	0.031	1.0	0.143	0.040
LGD*RPA waterbodies	97.0	0.316	0.038	1.0	0.137	0.041

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 1). Over all populations, we detected linkage disequilibrium for 27 out of 45 loci pairs (see Table S3). 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 Fig. S5 and Table 4). 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 $K = 7$ (Fig. 2).

Table 4: Expected and observed heterozygosity calculated with the repeated randomized selection of genotypes (RRSG) approach over all loci for breeding pond populations P1–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 5: Results of the repeated randomized selection of genotypes (RRSG) approach for the median pairwise FST (MPF) and median pairwise RST (MPR). Populations 1–6 were located within vineyards, populations 7–10 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	–

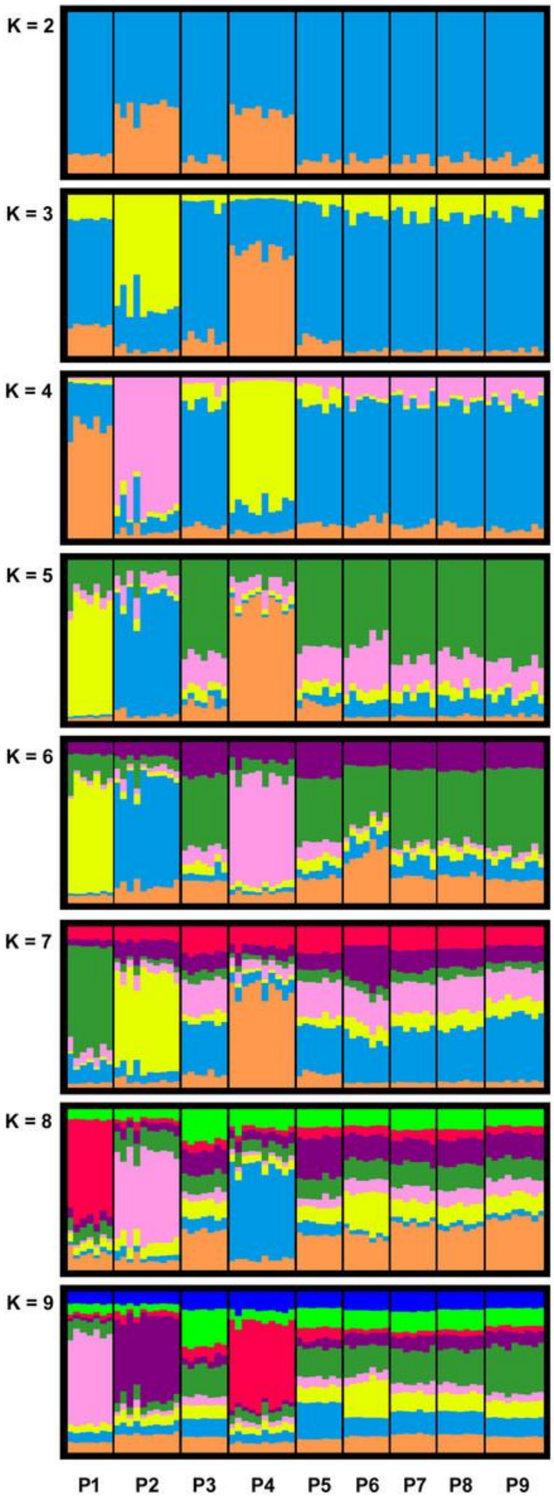

Fig. 2: Bar plots of combined STRUCTURE analysis for clusters K = 2 to K = 9 of the investigated R. temporaria breeding pond populations in the study area. STRUCTURE HARVESTER identified K = 4 as the most meaningful number of clusters. Each vertical bar represents one individual, and the color composition 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.

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 4). MPFs ranged from 0.0012 to 0.0987 and MPRs from 0.0000 to 0.1403 (Table 5). The highest MPF and MPR were estimated between P1 and P2 at a linear geographic distance of 2.4 km (see Table S5 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 5).

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 3). 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 Tables 2 and 3) 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).

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 metapopulation. 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 5) 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 (Fig. 2). 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 5). 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 5), 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 (Van der Sluis & Vos, 1997; Arens et al., 2007). Additionally, breeding sites in Noord-Brabant became polluted with agrochemicals (pesticides and fertilizers) as a result of intensive agriculture (Hoogerwerf & 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 (Buskirk, 2012; Beebee, 2013; Krug & 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 5). 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, Fig. 1) 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 (Roßberg, 2009; Lenhardt et al., 2013). 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 (Rothermel, 2004; Baldwin, Calhoun & DeMaynadier, 2006). 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 (WestheideRieger, 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 & Clark, 2007; Wright, 1978) but may increase due to time-delay in genetic differentiation (Bossart & 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 & Lugon-Moulin, 2002). The genetic differentiations identified by MPF values were supported by the estimated MPR values (Table 5), 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 (Smalling et al., 2013; Smalling et al., 2015; Battaglin et al., 2016; Cusaac et al., 2016). 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, Comitini & Ciani, 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 (Le Lay et al., 2015; Frei et al., 2016). Also, some level of pesticide tolerance for amphibians from agricultural breeding pond populations was detected (Hua, Morehouse & Relyea, 2013; Hua et al., 2015). 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 & 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, Schulze & Meyer, 2004; Schlüpmann et al., 1996; Wolfbeck, Laufer & Genthner, 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; Table S6). 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.

Additional Information and Declarations

Competing Interests

The authors declare there are no competing interests.

Author Contributions

Patrick P. Lenhardt, Carsten A. Brühl and Kathrin Theissinger conceived and designed the experiments.

Patrick P. Lenhardt performed the experiments, wrote the paper, prepared figures and/or tables.

Patrick P. Lenhardt, Christoph Leeb and Kathrin Theissinger analyzed the data and contributed reagents/materials/analysis tools.

Carsten A. Brühl, Christoph Leeb and Kathrin Theissinger reviewed drafts of the paper.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Field experiments were approved by the ”Struktur- und Genehmigungsdirektion Süd Referat 42-Obere Naturschutzbehörde” (Approval Number: 42/553-254).

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as Supplemental Information 1.

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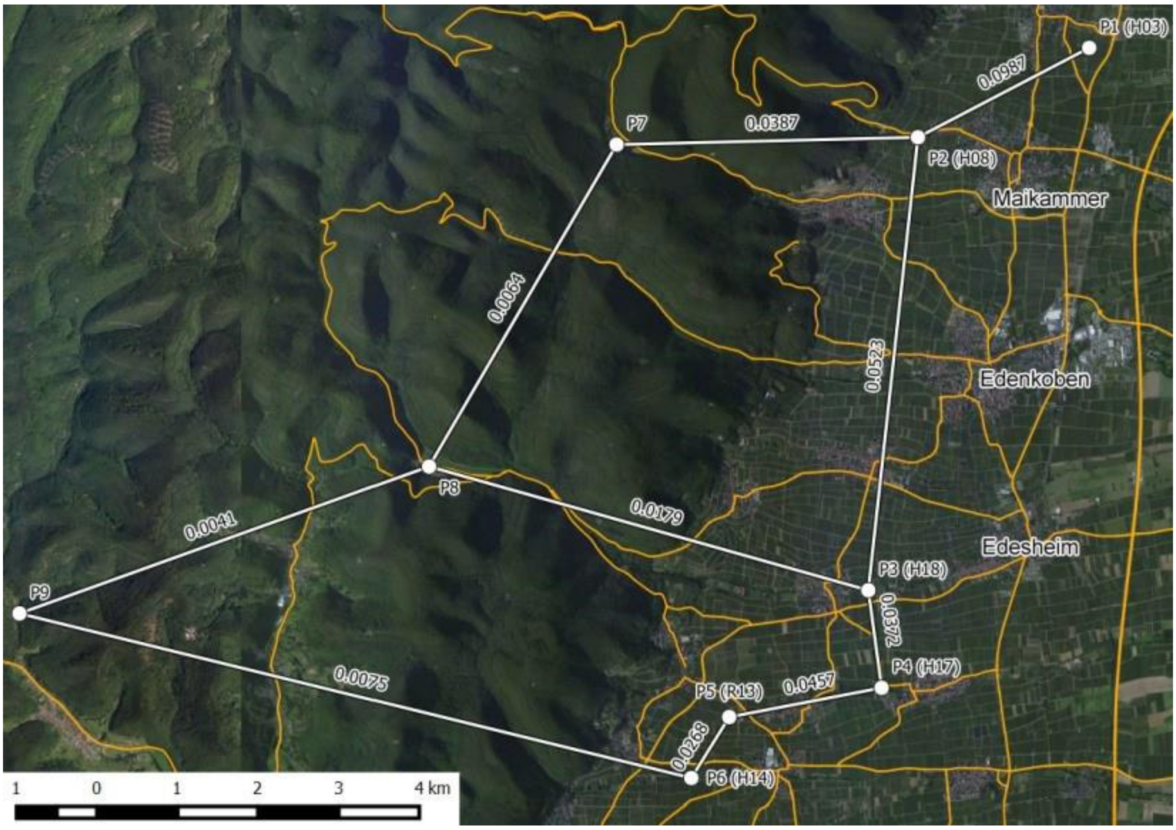
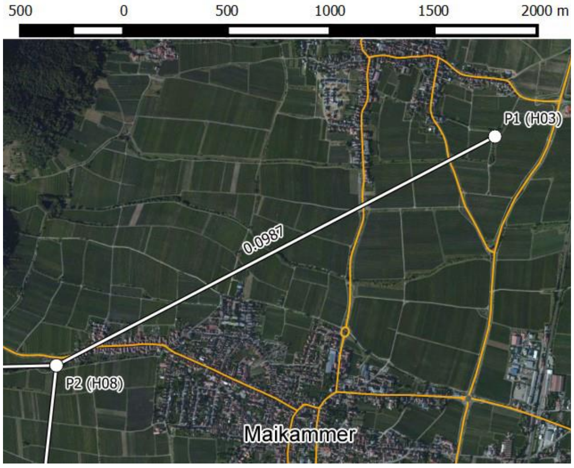
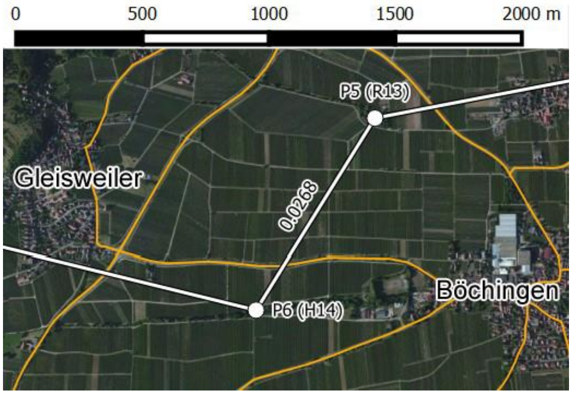
Supplemental Information



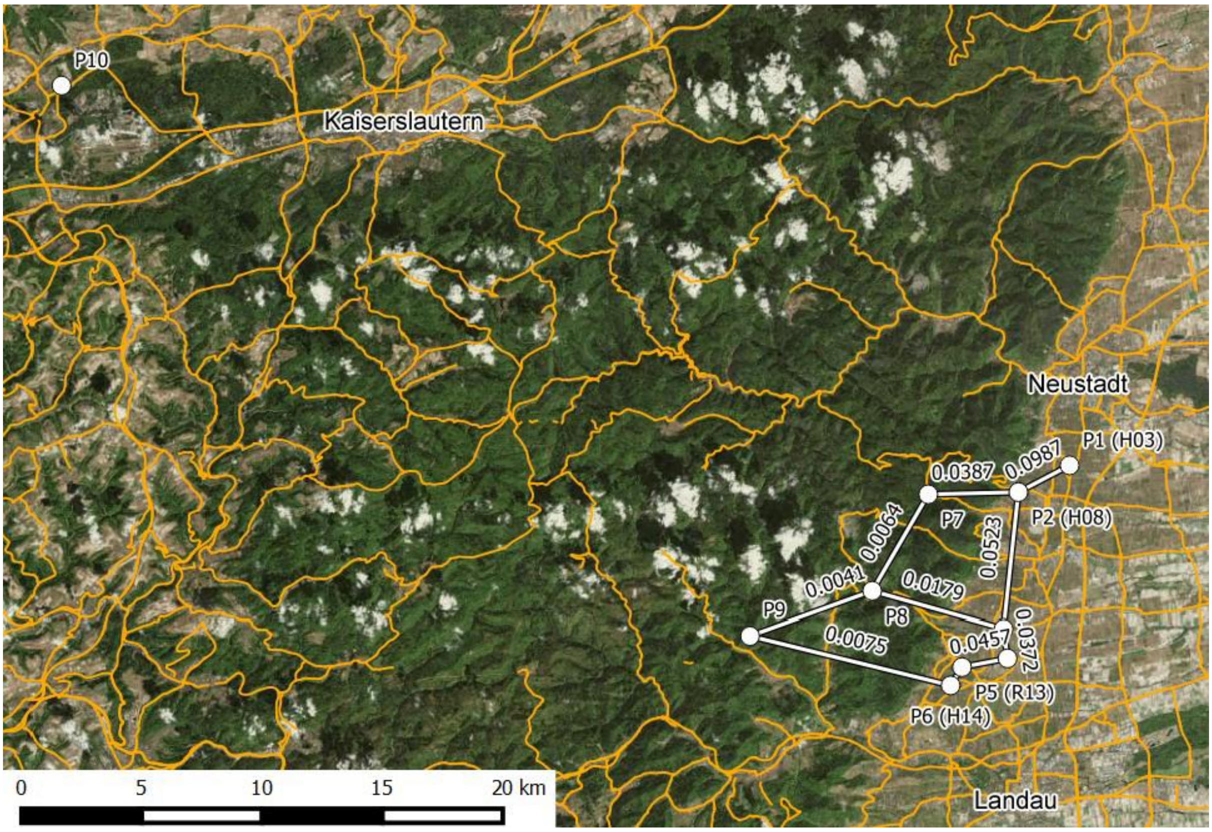
Appendix Fig. 1: Aerial photo of the core study area between “Neustadt (an der Weinstraße)” (north of P1) and “Landau (in der Pfalz)” (south of P6) with selected median pairwise FST values. Relevant traffic infrastructure is highlighted in orange (aerial photo from Bing Maps, http://www.bing.com/mapspreview).



Appendix Fig. 2: 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 FST value. Relevant traffic infrastructure is highlighted in orange (aerial photo from Bing Maps, http://www.bing.com/mapspreview).



Appendix Fig. 3: Close up aerial photo of landscape between breeding ponds P5 and P6 (890 m apart) with median pairwise FST value. Relevant traffic infrastructure is highlighted in orange (aerial photo from Bing Maps, http://www.bing.com/mapspreview).



Appendix Fig. 4: Aerial photo of the core study area between between "Neustadt (an der Weinstraße)" and "Landau (in der Pfalz)" with selected median pairwise FST values and P10 near Kaiserslautern. Relevant traffic infrastructure is highlighted in orange (aerial photo from Bing Maps, http://www.bing.com/mapspreview).

Appendix Table 1: 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	
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	*

Appendix Table 2: Number of roads between population pairs and their accumulated traffic intensity (vehicles in 24 hours on all roads between the pairs).

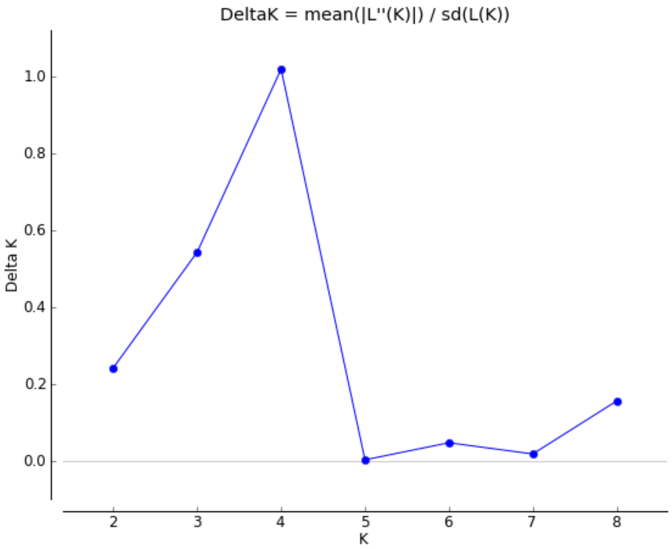
		P1	P2	P3	P4	P5	P6	P7	P8	P9
Number of roads	**P2**	3								
	P3	7	6							
	P4	10	7	2						
	P5	9	8	4	2					
	P6	10	9	5	3	1				
	P7	4	2	3	5	5	7			
	P8	6	3	4	7	5	5	1		
	P9	6	4	3	4	3	4	1	2	
	P10	14	15	15	18	15	15	15	12	12
accumulated traffic intensity	**P2**	4645								
	P3	14192	12978							
	P4	17165	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

Appendix Table 3: 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
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

Appendix Table 4: 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	-33.277.386	431.840	NA	NA	NA
2	500	-32.915.598	495.511	36.178.800	12.029.200	0.242764
3	500	-32.674.102	562.733	24.149.600	30.581.800	0.543451
4	500	-32.738.424	864.383	-6.432.200	88.074.400	1.018.928
5	500	-33.683.490	1.393.570	-94.506.600	0.514200	0.003690
6	500	-34.633.698	1.728.867	-95.020.800	8.314.400	0.048092
7	500	-35.667.050	2.055.675	-103.335.200	3.897.000	0.018957
8	500	-36.661.432	3.099.213	-99.438.200	48.534.200	0.156602
9	500	-37.170.472	3.377.702	-50.904.000	NA	NA



Appendix Fig. 5: Plot of delta K values from the Structure analyses, obtained through STRUCTURE HARVESTER.

Appendix Table 5: 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

Appendix Table 6: Clutch counts at the monitored amphibian breeding sites with presence of *Rana temporaria* during breeding seasons 2011 to 2014 (na = not available).

Pop.	2011	2012	2013	2014
P1	8	7	7	0
P2	10	12	0	8
P3	9	11	8	0
P4	19	22	18	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

Chapter 4

Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape

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Abstract

Amphibian populations are declining worldwide at alarming rates. Among the large variety of contributing stressors, chemical pollutants like pesticides have been identified as a major factor for this decline. Besides direct effects on aquatic and terrestrial amphibian stages, sublethal effects like impairments in reproduction can affect a population. Therefore, we investigated the reproductive capacity of common toads (*Bufo bufo*) in the pesticide-intensive viticultural landscape of Palatinate in Southwest Germany along a pesticide gradient. In a semi-field study, we captured reproductively active common toad pairs of five breeding ponds with different pesticide contamination level and kept them in a net cage until spawning. Toads from more contaminated ponds showed an increased fecundity (more eggs) but decreased fertilization rates (fewer hatching tadpoles) as well as lower survival rates and reduced size in Gosner stage 25, suggesting that the higher exposed populations suffer from long-term reproductive impairments. In combination with acute toxicity effects, the detected sublethal effects, which are mostly not addressed in the ecological risk assessment of pesticides, pose a serious threat on amphibian populations in agricultural landscapes.

Keywords

Amphibians, Semi-field study, Fecundity, Population decline, Sublethal effects

Introduction

The latest IUCN reports suggest that 41% of all amphibian species are threatened (IUCN 2020). Besides habitat modification and destruction, intensive agriculture including the exposure to chemical pollutants like pesticides is one of the major factors for the global amphibian decline (Collins and Storfer 2003; Stuart et al. 2004). Several studies investigating the impact of intensive agriculture on amphibians determined adverse effects on egg and tadpole health (Babini et al. 2018), adult body condition, and morphology (Bionda et al. 2018; Hegde et al. 2019; Zhelev et al. 2017). One reason for these effects can be the exposure of amphibians to pesticides, with which they can come into contact during their whole life cycle. They can be exposed during the breeding phase and larval development in their aquatic habitats due to spray-drift (Crossland et al. 1982), run-off (Edwards et al. 1980) and drainages (Brown and van Beinum 2009). Post-metamorphic, terrestrial juvenile and adult amphibians can take up pesticides e.g., from contaminated soil (Storrs Méndez et al. 2009) during migration through the agricultural landscape (Leeb et al. 2020b; Lenhardt et al. 2013). Despite this chronic, biphasic exposure, the effects of chemical pollutants on amphibian declines is not well understood (Grant et al. 2016). Most ecotoxicological laboratory studies on amphibians focus on acute effects of pesticides that lead to direct mortality in aquatic or, more rarely studied, terrestrial life stages (e.g., Brühl et al. 2013; Relyea 2004, 2005). Besides these acute effects, chronic and sublethal effects due to impaired reproduction may also result in amphibian population declines. Thus, there is not only a potential for rapid but also long-term amphibian declines, either due to impairment of adult breeding or deficient development of a progeny (Hayes et al. 2010b).

On the one hand, sublethal effects on reproduction can occur due to direct systemic toxicity. Effects on molecular biomarkers like acetylcholine esterase activities (Hegde et al. 2019) and hematological parameters (Zhelev et al. 2018) as well as genotoxic and mutagenic effects (Gonçalves et al. 2019) may have an impact on the reproductive capacity and thus on amphibian populations. Moreover, resources for the production of eggs may be limited and reproduction reduced due to resources required for pesticide detoxification processes as shown for the woodlouse Porcellio scaber (Jones and Hopkin 1998). Pesticides may also indirectly affect amphibian reproduction by interfering with their food supply (Sánchez-Bayo and Wyckhuys 2019) or affecting their behavior and thus disturbing their habitat use (Leeb et al. 2020a), predation (Adams et al. 2020), mating behavior (Schwendiman and Propper 2012) and population connectivity (Lenhardt et al. 2017).

On the other hand, pesticides can also directly act on the hormonal pathways of developmental processes as endocrine disrupting chemicals (EDCs), which alter the normal functioning of the endocrine system leading to impaired reproduction mechanisms such as infertility or intersex (Ujhegyi and Bókony 2020). EDCs have been found in amphibian breeding sites in agricultural landscapes. Bókony et al. (2018) detected 41 EDCs across amphibian ponds in the agricultural landscape of Hungary. Müller and Zithier (2015) performed a monitoring of ten pesticides in small water bodies used by amphibians in agricultural landscapes in North Germany and detected amongst others the potential EDCs metazachlor and propiconazole. However, in general little information on pesticide contamination is available on water bodies used by amphibians for spawning and larval development, as most studies investigate pollution of groundwaters, river systems and lakes (Lorenz et al. 2017), neglecting small, shallow water bodies that are especially important for amphibians (Wells 2007).

Studies on direct reproduction effects of pesticides on amphibians are considerably rare. One of the few well-studied pesticides with endocrine disruptive properties is the insecticide atrazine that shows severe effects on the reproduction of amphibians. Larvae of African clawed frogs (*Xenopus laevis*) showed a decreased gonadal volume and germ cells (Tavera-Mendoza et al. 2002a, b) as well as a trend to hermaphroditism (Hayes et al. 2002b) after exposure to atrazine. Further, atrazine induced feminization of male leopard frogs (*Lithobates pipiens*) in nature (Hayes et al. 2002a). Pesticide mixtures containing atrazine also indirectly inhibit reproductive functioning, e.g., by increasing stress hormone levels like corticosterone in adult *X. laevis* (Hayes et al. 2006). This may lead to further impacts including inhibition of sex hormones (Burmeister et al. 2001) and the alteration of reproductive development, breeding behavior and fertility (Moore 1983). Other current-use pesticides with endocrine disruptive properties are for example dicarboxamides like the viticultural fungicide vinclozolin (Kortekamp et al. 2011). This fungicide has been shown to contribute to shifted sex ratios, an inhibited maturation and reduced fecundity as well as fertility in fish (Lor et al. 2015). Although a few studies have explored endocrine disrupting effects of viticultural azole fungicides like tebuconazole and penconazole (e.g., Lv et al. 2017; Poulsen et al. 2015), they are not yet considered as EDCs by the Pesticide Properties DataBase (PPBD, Agriculture and Environment Research Unit of the University of Hertfordshire 2013) and the PAN International List of Highly Hazardous Pesticides (PAN List of HHPs; Pesticide Action Network International 2019). Further pesticides may have similar effects, however, the database on endocrine disruptive properties is too small to allow for concrete conclusions.

Especially field data on sublethal reproduction endpoints are scarce because mainly laboratory studies are used to investigate effects of pesticides on reproduction. Thereby, the most investigated endpoint in field studies analyzing effects on reproduction is the incidence of intersex, in which individual´s gonads contain both female and male tissue (Ujhegyi and Bókony 2020). However, also other endpoints like the number of laid eggs, fertilization rates or the development success of early larvae can be used to evaluate effects of pesticides on the reproductive capacity. Bókony et al. (2018) investigated the effects of EDCs on common toads (*Bufo bufo*) in agricultural and urbanized ponds in Hungary and observed reduced developmental rates and lower body mass of the offspring compared to natural ponds.

Investigations on pesticide effects on the reproduction of amphibians in viticultural landscapes do not exist so far, although viticulture is one of the most pesticide-intensive cultures in Central Europe. On average 9.5 pesticide applications with a mixture of on average 1.6 formulations per application are performed during March and August in vineyards (Roßberg 2009). Because of the combined aquatic and terrestrial exposure of amphibians to viticultural pesticides, long-term adverse effects on reproduction are likely. To address this lack of knowledge, we investigated the reproductive capacity of common toads (*Bufo bufo*, LINNAEUS 1758) in the viticultural landscape of Palatinate in Southwest Germany along a pesticide gradient. We hypothesized that an increased chronic pesticide exposure affects fecundity, fertilization rate as well as offspring survival and size. Common toads were used since it is the most common amphibian species in Central Europe (Sillero et al. 2014) and it occupies a broad range of habitat types including agricultural landscapes like vineyards (Leeb et al. 2020b; Lenhardt et al. 2013). They are not yet considered endangered on an international as well as national level (Agasyan et al. 2009; Kühnel et al. 2009). However, population declines have been observed on a local level (e.g., Beebee and Griffiths 2005; Bonardi et al. 2011; Kyek et al. 2017; Petrovan and Schmidt 2016).

Materials and methods

Study sites and exposure assessment

In spring 2019, we studied common toad populations from five ponds (pond A–E, Table 1, Fig. 1) around Landau, one of the largest winegrowing areas in Southwest Germany. These ponds were expected to represent a gradient of pesticide contamination due to their varying agricultural surrounding. For validation of the pesticide gradient, five water samples were collected of each pond between April and May 2018 and analyzed for 47 different fungicides, six insecticides, three herbicides, and two acaricides (Table S1) by the Institute of Phytomedicine of the Dienstleistungszentrum Ländlicher Raum Rheinpfalz in Neustadt/Weinstraße, Germany. The selection of analyzed pesticides was based on spraying recommendations for vine from local authorities (www.dlr.rlp.de).

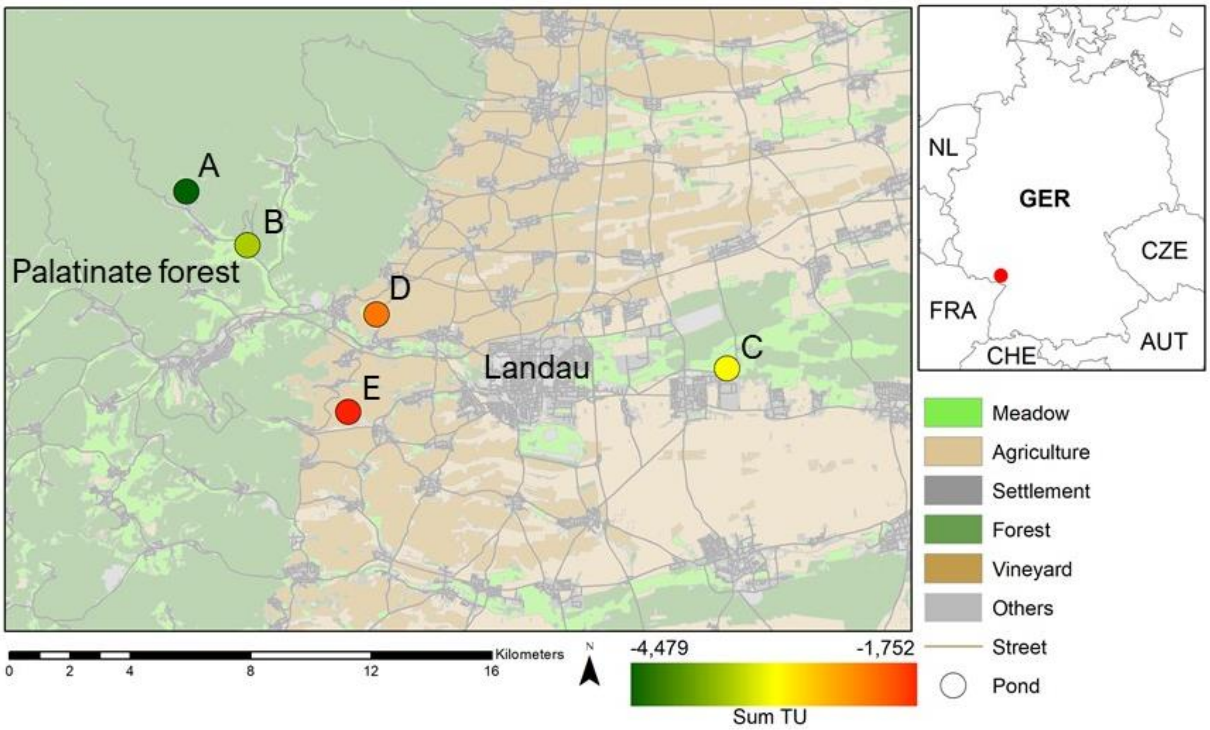

Fig. 1: Map of study ponds in Palatinate in Southwest Germany. Increasing letters and colors of study sites represent the pesticide contamination from no contamination (dark-green, pond A) to high contamination (red, Pond E). Source: Basemap: DLM50 - ©GeoBasis-DE / LVermGeoRP2020, dl-de/by-2-0, www.lvermgeo.rlp.de [modified data].

Table 1: Locations of study ponds, contamination level (sum of toxic units, STU, see Equation 2), number of captured toad pairs and number of toad pairs that spawned. Pond letters indicate increasing STU. Since no pesticides were detected in pond A, its STU was calculated based on the use of 1/10 of the minimum TU observed in the sites with detected concentrations (for rationale s. Schäfer et al. 2011)

Pond	Coordinates (WGS84)	STU	Number of toad pairs	
			Captured	Spawned
A	49.25475, 7.96182	-4.48	12	11
B	49.23830, 7.99002	-3.48	13	11
C	49.20329, 8.20917	-3.09	15	13
D	49.21830, 8.04944	-2.25	14	14
E	49.18898, 8.03709	-1.75	8	5

The pond pesticide toxicity was assessed using Toxic Units for each detected pesticide (Eq. 1, with C_i = detected concentration of pesticide *i* and LC50$_i$ = median lethal concentration causing 50% mortality of test organisms).

$$\mathrm{TU} = \frac{\mathrm{C_i}}{\mathrm{LC50_i}} \qquad (1)$$

As LC50 values for amphibians are often lacking, data of acute fish toxicity studies compiled from the PPDB (Agriculture and Environment Research Unit of the University of Hertfordshire 2013) were used as proxy for amphibians (Weltje et al. 2013). The sum of TU (STU, Eq. 2, with *n* = number of detected pesticides) was calculated to aggregate the toxicity of the detected pesticides (Table 1, Schäfer et al. 2011) by using the maximum detected sum of TU of each study pond. To allow the comparison to sites without any detected pesticides, uncontaminated ponds were assigned to a TU of 1/10 of the minimum TU observed in the contaminated sites (Fernández et al. 2015), leading to a STU of −4.48 for pond A.

$$\mathrm{STU} = \log(\max \textstyle\sum_{i=1}^{n} \mathrm{TU}) \qquad (2)$$

The detected pesticides were checked for endocrine disruptive properties using toxicity data from the PPDB (Agriculture and Environment Research Unit of the University of Hertfordshire 2013) and the PAN List of HHPs (Pesticide Action Network International 2019). Moreover, acute and chronic regulatory acceptable concentrations (RACs) were calculated based on fish toxicity values from the PPDB (LC50 and NOEC = No observed effect concentration, Eqs. 3, 4, Table S2). As uncertainty factors, 100 was used for the acute and 10 for the chronic RAC as recommended for aquatic organisms by EFSA (2013). The RACs were compared to the detected concentrations to estimate the acute and chronic aquatic toxicity of the ponds.

$$RAC_{acute} = \frac{LC50}{100} \quad (3)$$

$$RAC_{chronic} = \frac{NOEC}{10} \quad (4)$$

Moreover, the landscape composition around the study ponds was analyzed. Based on a vector landscape model of Rhineland-Palatinate (ATKIS DLM50), the percentages of vineyards, other agriculture, meadows, settlements, and forests were calculated. A radius of three kilometer was chosen to analyze the landscape composition because this distance reflects the annual migrations between hibernation as well as summer habitats and breeding ponds for *B. bufo* (Günther 2009). To estimate the terrestrial exposure, data of viticultural and other agricultural area was used.

Reproductive capacity analysis

We aimed to capture ten or more reproductively active adult common toad pairs during their spawning season between 9 and 28 March 2019 from each pond. After capturing, each pair was housed in a net cage (80 × 65 × 60 cm) in the respective breeding pond containing a wire hanger as spawning substrate. Due to the short spawning season of *B. bufo* and the fact that not all pairs spawned, it was not possible to investigate ten spawning pairs of each pond (Table 1). Finally, we captured 62 toad pairs from which eight pairs did not spawn, 45 pairs spawned within 7 days and nine pairs within 15 days after catchment. One day after spawning, the body mass of each toad was measured (±0.1 g) and the individuals were released in their ponds. It can be assumed that females laid all eggs at once because the spawning process is usually finished after 6 to 12 h (Günther 2009) and the pairs terminated the amplexus after oviposition.

As measures of each population's reproductive capacity, we analyzed the fecundity, fertilization rate, offspring survival until the free-swimming Gosner Stage 25 (GS; Gosner 1960) and offspring size (tadpole length) at GS25. To determine the fecundity, the number of laid eggs per female was counted. Because fecundity is known to increase with female size (Banks and Beebee 1986; Reading 1986), we calculated the ratio of the amount of laid eggs and the body mass of the females after spawning (eggs/g body mass). To estimate the fertilization rate and offspring survival, approximately 90 eggs of each clutch were removed from three randomly chosen parts of the egg string and kept individually in clear plastic aquariums (22.5 × 16.5 × 7 cm, Braplast, Bergheim, Germany) filled with 1 L FETAX medium (Dawson and Bantle 1987). To prevent any injuries of eggs, the handling of the spawning strings was kept to a minimum. Thus, the number of eggs was not identical for each sample. Because mold grew on the first three egg strings collected from pond C, three samples of pond C could not be used to analyze the fertilization rate and offspring survival. To prevent mold from growing on further eggs, eggs of one egg string were separated but still incubated together in one aquarium. The eggs were reared in a climate chamber at 21 ± 1 °C and a 16:8 h day:night light cycle until they reached GS25. The individuals were photographed daily. Three days after spawning, non-fertilized eggs that exhibited mold growing on them or did not show embryonic development were removed. Developing eggs were counted using Image J (Schneider et al. 2012) to calculate the fertilization rate. Fertilized eggs from one egg string hatched within a time difference of maximum 24 h. As soon as all tadpoles reached GS25 (9–10 days), the proportion of embryos that survived to this stage was counted to estimate the offspring survival. Moreover, the lengths of twelve randomly selected tadpoles of each sample were determined to estimate the offspring's sizes. After recording the needed data, the tadpoles were released in their origin pond.

Statistical analyses

Statistical analyses were performed using R (version 3.5.2; R Core Team 2013). To determine the correlation of the aquatic and terrestrial exposure, a Pearson´s correlation was performed. Kendall-Theil Sen Siegel non-parametric regressions (Sen 1968; Siegel 1982; Theil 1950) were performed to check whether the investigated endpoints depend on the pesticide contamination of ponds (STU). Moreover, Spearman's rank correlations between the investigated endpoints and the STU of ponds were computed (Spearman's rank correlation coefficient ρ, Hollander et al. 1973).

To check the assumption that fecundity is increased by female size, a Spearman's rank correlation was performed for the female body mass and the number of laid eggs. Moreover, Spearman's rank correlations were performed to investigate the relationship between the pesticide contamination (STU) and the female body mass, the number of laid eggs and the tadpole length in GS25, parental body masses and the fertilization rate as well as the number of laid eggs per female and the fertilization rate. To investigate a measure of population fitness, the product of the four investigated reproductive endpoints was calculated and a one-way analysis of variance (ANOVA) was performed to identify differences between the investigated ponds. Tukey's method was used to identify and remove outliers ranged above and below the 1.5 × IQR (Kannan Senthamarai et al. 2015). For all statistical tests, the criterion for significance was set to $\alpha = 0.05$.

Results

Exposure assessment

The pesticide residue analysis revealed 22 different pesticides in total and 0–19 different pesticides per pond with a STU between −4.48 and −1.75 (Tables 1, S2) meaning no aquatic toxicity at a STU of −4.48 and high toxicity at a STU of −1.75. Toxicity data extracted from the PPDB and the PAN List of HHPs for the detected pesticides did not show any endocrine disruptive properties or the data base was insufficient to make a statement about endocrine disruptive properties. However, azole fungicides which were shown to be potential EDCs (Kortekamp et al. 2011; Lv et al. 2017; Poulsen et al. 2015) were detected in the ponds. Penconazole was detected in ponds B, D and E (0.02–0.18 µg/L), tebuconazole in ponds C, D and E (0.05–0.08 µg/L) and difenconazole in pond C (0.02 µg/L).

The comparison of detected concentrations to RACs revealed a conspicuous toxicity of the chronic exposure to the fungicides folpet and famoxadone and the acute exposure to famoxadone in pond E (Table S2). The chronic RAC of folpet was 5.6 times lower than the detected concentration in sampling 2 (4.53 µg/L), the chronic RAC of famoxadone was 1.1 times lower and the acute RAC of famoxadone was 1.4 times lower than the detected concentration in sampling 5 (0.15 µg/L), resulting in an increased hazard of adverse effects.

The landscape composition analysis showed an increasing agricultural land-use from pond A to pond E in a three-kilometer radius around the study ponds ranging from 0 to 60% (Table 2). The Pearson correlation revealed a statistically significant correlation between the STU and the agricultural land-use ($p = 0.02$, Pearson's $r = 0.94$, df = 3).

Table 2: Landscape composition in a radius of 3000 m around the study ponds based on a vector landscape model of Rhineland-Palatinate (ATKIS DLM50)

Pond	Viticulture [%]	Other agriculture [%]	Meadow [%]	Settlement [%]	Forest [%]	Other [%]
A	0.0	0.0	5.1	1.3	92.9	0.6
B	0.1	1.1	19.2	5.6	72.1	1.9
C	0.3	31.4	19.6	15.5	28.5	4.8
D	47.5	1.1	7.9	11.6	29.8	2.2
E	57.0	3.1	6.1	10.1	22.5	1.3

Reproductive capacity

Neither the female body mass (52.0 ± 14.1 g), the male body mass (33.46 ± 6.7 g), nor the number of laid eggs per female (3243 ± 1538) affected the fertilization rate ($\rho = -0.24$, $p = 0.10$, $\rho = -0.09$, $p = 0.56$ and $\rho = -24$, $p = 0.10$). The female body mass was positively correlated with the number of laid eggs ($\rho = 0.62$, $p < 0.001$) and the STU ($\rho = 0.38$, $p < 0.01$). Moreover, the offspring size (tadpole length in GS25) was negatively correlated with the number of laid eggs per female ($\rho = -0.32$, $p = 0.03$).

Kendall-Theil Sen Siegel regressions revealed a significant influence of the STU on all investigated endpoints ($p < 0.001$, Table S3). The mean fecundity differed from 49 to 74 eggs/g body mass and showed a positive correlation with increasing STU ($\rho = 0.54$, $p < 0.001$, Fig. 2A, Table S4). The fertilization rate, offspring survival and tadpole lengths showed mean decreases of 4.5%, 32.6% and

10.7% with increasing STU (Fig. 2A–D, Table S4). Negative correlations between the STU and the fertilization rate ($\rho = -0.32$, $p = 0.03$, Fig. 2B), the offspring survival ($\rho = -0.57$, $p < 0.001$, Fig. 2C) as well as the offspring size ($\rho = -0.49$, $p < 0.001$, Fig. 2D) were observed. The performed ANOVA did not reveal any differences for population fitness between the study ponds ($p > 0.05$).

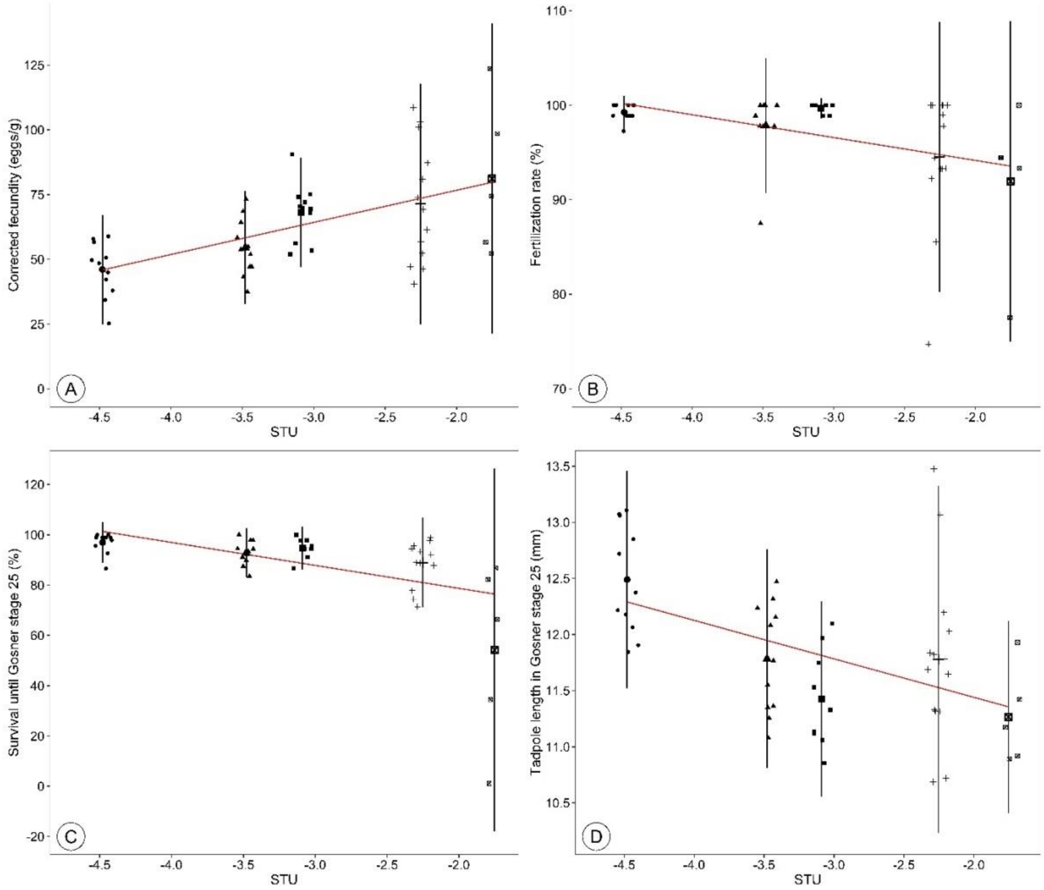

Fig. 2: Dependence of fecundity (A), fertilization rate (B), offspring survival until Gosner stage 25 (C) and offspring size in Gosner stage 25 (D) on the pesticide contamination of breeding ponds (maximum sum of toxic units, STU). Fecundity was corrected for the body mass of the females after spawning (eggs/g body mass). For each pond, the means and standard deviations are presented (Table S4).

Discussion

Exposure assessment

Since pesticide contamination of ponds are often reported to correlate with the surrounding agricultural land-use (Baker 2006), it was assumed that the detected pesticide gradient also represents the exposure during the pre- and post-breeding migration of the terrestrial amphibian stages. The determined correlation of aquatic exposure and land-use confirms this hypothesis.

No general statement can be drawn about the endocrine disruptive potential of the detected pesticides because further research is needed on their potential to act as EDCs. The well-studied endocrine disrupting herbicide atrazine was not detected in any of the study ponds probably because it is prohibited in Germany since 1991. However, since potentially endocrine disruptive pesticides like the azole fungicides penconazole, tebuconazole and difenconazole were detected, similar endocrine effects are likely. Furthermore, the ponds were only analyzed for active ingredients of pesticides. A statement about the toxicity of product additives, which can have a high acute toxicity, endocrine disruptive or reproductive toxic properties themselves or as metabolite (Mesnage and Antoniou 2017; Mullin et al. 2016), cannot be made.

The comparison of detected concentrations to chronic RACs of folpet and famoxadone in pond E reveals a high toxicity for aquatic vertebrates. Next to possible adverse effects because of single pesticides, mixture effects in ponds with up to 19 detected pesticides may contribute to higher toxicities (Relyea 2009). Moreover, it cannot be excluded that even higher concentrations and further pesticides were present in the ponds due to the limited number of water samplings ($n = 5$) and analyzed pesticides ($n = 58$ target molecules). Since only one rain event sampling was performed in the present study, peak pesticide concentrations may be underestimated (Neumann et al. 2003). Especially folpet and famoxadone may be present at higher concentrations than detected because they have very short dissipation times in water (DT50 folpet = 0.02 d, DT50 famoxadone = 0.1 d, Agriculture and Environment Research Unit of the University of Hertfordshire 2013).

Reproductive capacity

Toads of the highest contaminated pond E showed on average a 1.5 times higher fecundity than toads of the uncontaminated pond A. In comparison to the present study, Bókony et al. (2018) did not observe any effect on the fecundity of common toads in agricultural ponds compared to natural ponds. Because the female body mass correlated with the number of eggs and both of them correlated with STU, the increased fecundity may be based on the higher female body masses in the contaminated ponds. Guillot et al. (2016) also observed larger and heavier common toads in French agricultural habitats compared to uncontaminated forest habitats. The increased body sizes might either suggest a potential adjustment during aging or some habitat specificities in the agricultural landscape may enhance body size. For example, smaller population densities in agricultural landscapes might decrease intra- and/or interspecific competition leading to larger individuals (Bishop et al. 1999; Guillot et al. 2016; Janin et al. 2011). However, there are multiple reasons that may affect adult body size without an agricultural context such as climate, habitat geography, size at metamorphosis, and availability of food resources.

The fertilization rate was negatively affected with increasing pesticide contamination of the ponds, suggesting that the higher exposed populations suffer from long-term reproductive impairments. There are several reasons that may have led to the observed decreased fertilization rate. Due to the increased number of eggs per female, the male fertilization success may be reduced. But also behavioral impairments during mating could lead to decreased fertilization rates. Hayes et al. (2010a) observed a reduced success of amplexus in male *X. laevis* exposed to atrazine and thus a lower proportion of fertilized eggs for atrazine exposed males. Also endocrine disruptive properties of pesticides may have led to this decrease for example due to impaired spermatogenesis which already has been reported after the exposure of frogs to the herbicide atrazine. Hayes et al. (2010a) observed a decreased frequency of testicular tubules with mature spermatozoa in *X. laevis*. In *X. laevis* tadpoles a reduction in testicular volume during sexual differentiation of the testis was observed (Tavera-Mendoza et al. 2002b). Another reason may be an effect on female sexual development. In-vitro assays with eleven pesticides of Orton et al. (2009) revealed altered ovarian steroidogenesis and reduced progesterone production. Pickford and Morris (2003) investigated the effects of the insecticide methoxychlor on female *X. laevis* and detected an inhibition of oviposition and maturation of oocytes. Moreover, the exposure to atrazine caused a reduction in the number of germ cells in the ovary and an increase of damaged oocytes (Tavera-Mendoza et al. 2002b). The larval exposure of *X. laevis* to atrazine induced a reduction of testosterone levels in males (Hayes et al. 2010a) leading to a decrease of male reproductive success (Moore and Hopkins 2009).

Decreasing survival rates and tadpole sizes were observed with increasing pesticide contamination. Bókony et al. (2018) also observed reduced body masses of common toad larvae and juveniles in agricultural landscapes in comparison to natural landscapes. Clearly, decreased survival of the tadpoles directly leads to population declines. The reduced tadpole lengths could lead to further impairments since body size is a critical determinant of individual fitness (Wells 2007). Smaller tadpoles sizes lead to reduced sizes at metamorphosis and thus to a decreased survivorship of the first hibernation (Üveges et al. 2016) and until maturity as well as delayed achievement of reproductive size (Smith 1987). Reduced body size is also a disadvantage as adult for reproduction because it affects female fecundity and male mating success (Banks and Beebee 1986; Davies and Halliday 1979; Reading et al. 1991).

On the one hand, reduced offspring size may be a long-term consequence of chronic pesticide pollution over several generations. Transgenerational effects were observed in rats after the exposure to EDCs as Anway et al. (2005) detected a decreased spermatogenic capacity in cell number and viability as well as an increase of male infertility in four tested generations. Thus, early-life exposure of parents can lead to impaired offspring viability. To verify the proposed reasons of reproduction impairments regarding endocrine disruptive effects, tissue analyses of e. g. thyroids and gonads would be needed. However, the present study was designed and completed without any lethal interferences and tissue withdrawals of the amphibian populations.

On the other hand, the reduced offspring size originating from highly contaminated ponds may be a cost of an evolutionary adaptive resistance (Whitehead et al. 2012) or of detoxification processes of contaminants (Rix et al. 2016). Similar effects have been observed for urban fish populations which evolved tolerance to toxic pollutants (Meyer and Di Giulio 2003; Whitehead et al. 2012). However, their offspring showed reduced growth rates and were more susceptible to other stressors compared with the offspring from a non-contaminated site (Meyer and Di Giulio 2003). Similar trade-offs may be responsible for the smaller tadpoles of the more contaminated ponds. Adult toads of these ponds may invest more resources into the production of egg jelly coat material to provide a better protection against pesticides. These resources may have in turn not be invested into larger ova (Podolsky 2004) which may have led to smaller tadpoles such as determined by Kaplan (1980). The higher egg production in contaminated ponds may be discussed as an adaptation to increase fitness by counterbalancing negative pesticide effects on embryo and tadpole development by an increased egg number.

Although amphibians are especially affected by pesticides due to their biphasic lifecycle, they are not yet considered in the environmental risk assessment of pesticides in the EU (Ockleford et al. 2018). Our data support the suggestion of inhibitory effects of current-use pesticides on the reproductive capacity of amphibians, potentially contributing to population declines. Thus, not only acute effects should be investigated in ecotoxicological amphibian studies but also sublethal effects on reproduction on a population level. Since data involving field scenarios analyzing the effects of multiple pesticides on amphibian reproduction are considerably rare, our results are of significant importance for amphibian conservation in agricultural landscapes.

Additional Information and Declarations

Data availability

Data are available by contacting EA (adams@uni-landau.de).

Code availability

The used R code is available by contacting EA (adams@uni-landau.de).

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Author contributions

All authors conceived and designed the study. EA performed the study, analyzed the data and drafted the manuscript. CL generated the map. CL and CAB contributed to and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was approved by the Struktur- und Genehmigungsdirektion Süd (Neustadt an der Weinstraße, Germany, license number 42/553-254/457-19).

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Supporting information

Table S1: Investigated target pesticides of the aquatic residual analysis. In total 47 different fungicides, six insecticides, three herbicides and two acaricides were investigated.

Fungicides	Insecticides	Herbicides	Acaricides
Amisulbrom Azoxystrobin Benalaxyl-M Benthivalicarb Boscalid Captan Cyazofamid Cyflufenamid Cyprodinil Dichlofluanid Difenoconazole Dimethomorph Epoxiconazole Famoxadone Fenarimol Fenhexamid Fenpropimorph Fenpyrazamine Folpet Fludioxonil Fluopicolide Fluopyram Fluquinconazole Iprodion Iprovalicarb Kresoxim-methyl Metalaxyl M Metrafenone Myclobutanil Penconazole Prochloraz Procymidon Propinconazole Proquinazid Pyraclostrobin Pyrifenox Pyrimethanil Quinoxyfen Spiroxamin Tebuconazole Tetraconazole Tolylfluanid Triadimefon Triadimenol Trifloxystrobin Vinclozolin Zoxamide	Chlorpyrifos-methyl Dimethoate Indoxacarb Methidathion Parathion-ethyl Parathion-methyl	Atrazine Carfentrazone-ethyl Simazine	Spirodiclofen Tebufenpyrad

Table S2: Detected pesticide concentrations in the aquatic residual analysis of each study pond and respective risk assessment parameters. In total five samplings were performed (06.04.2018, 19.04.2018, 03.05.2018, 10.05.2018 - rain event, 24.05.2018). Pond letters indicate increasing pesticide contamination (based on the sum of toxic units). DC = Detected concentration; NOEC = No observed effect concentration (chronic toxicity) for fish and LC50 = Median lethal concentration causing 50% mortality of fish (acute toxicity) were extracted from the Pesticide Properties Database (Agriculture and Environment Unit of the University of Hertfordshire 2013); NOEC-RAC = Regulatory acceptable concentration based on the NOEC values, which are divided by the assessment factor for chronic studies (10); LC50-RAC = Regulatory acceptable concentration based on the LC50 values, which are divided by the assessment factor for acute studies (100). Detected concentrations were divided by calculated RAC values. A result > 1 (highlighted in bold) reveals a possible hazard for aquatic organisms.

Sampling	Pesticide	DC (µg/L)	NOEC (µg/L)	NOEC-RAC (µg/L)	DC/NOEC-RAC	LC50 (µg/L)	LC50-RAC (µg/L)	DC/LC50-RAC
Pond A								
1-5	-	-	-	-	-	-	-	-
Pond B								
1	Boscalid	0.09	125	12.5	0.0072	2700	27	0.0033
	Fludioxonil	0.04	40	4	0.0100	230	2.3	0.0174
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
2	Boscalid	0.02	125	12.5	0.0016	2700	27	0.0007
	Zoxamide	0.03	4	0.4	0.0750	160	1.6	0.0188
3	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
4	Fludioxonil	0.06	40	4	0.0150	230	0.23	0.26
	Myclobutanil	0.14	200	20	0.0070	2000	2	0.07
	Dimethoate	0.02	400	40	0.0005	30200	30.2	0.0007
5	Boscalid	0.02	125	12.5	0.0016	2700	27	0.0007
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Penconazole	0.02	360	36	0.0006	1130	11.3	0.0018
Pond C								
1	Azoxystrobin	0.04	147	14.7	0.0027	470	4.7	0.0085
	Difenconazole	0.02	23	2.3	0.0087	1100	11	0.0018
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Iprovalicarb	0.28	9890	989	0.0003	22700	227	0.0012
	Metalaxyl M	0.08	9100	910	0.0001	27000	270	0.0003
	Myclobutanil	0.03	200	20	0.0015	2000	20	0.0015
	Dimethoate	0.05	400	40	0.0013	30200	302	0.0002
2	Azoxystrobin	0.03	147	14.7	0.0020	470	4.7	0.0064
	Fluopyram	0.02	135	13.5	0.0015	42900	429	0.0000
	Zoxamide	0.12	4	0.4	0.3000	160	1.6	0.0750
3	Folpet	0.33	8.1	0.81	0.4074	680	6.8	0.0485
	Iprovalicarb	0.09	9890	989	0.0001	22700	227	0.0004
4	Azoxystrobin	0.02	147	14.7	0.0014	470	4.7	0.0043

	Fludioxonil	0.05	40	4	0.0125	230	2.3	0.0217
	Iprovalicarb	0.11	9890	989	0.0001	22700	227	0.0005
	Myclobutanil	0.04	200	20	0.0020	2000	20	0.0020
	Pyrimethanil	0.02	1600	160	0.0001	10560	105.6	0.0002
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
5	Cyflufenamid	0.02	24	2.4	0.0083	1040	10.4	0.0019
	Folpet	0.22	8.1	0.81	0.2716	680	6.8	0.0324
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Iprovalicarb	0.11	9890	989	0.0001	22700	227	0.0005
	Metrafenone	0.10	228	22.8	0.0044	820	8.2	0.0122
	Myclobutanil	0.10	200	20	0.0050	2000	20	0.0050
	Tebuconazole	0.07	10	1	0.0700	4400	44	0.0016
	Chlorpyrifos-methyl	0.02	5	0.5	0.0400	410	4.1	0.0049
	Dimethoate	0.04	400	40	0.0010	30200	302	0.0001
Pond D								
1	Dimethomorph	0.06	56	5.6	0.0107	6100	61	0.0010
	Famoxadone	0.06	1.4	0.14	0.4286	11	0.11	0.5455
	Iprovalicarb	0.44	9890	989	0.0004	22700	227	0.0019
	Metalaxyl M	0.08	9100	910	0.0001	27000	270	0.0003
	Myclobutanil	0.22	200	20	0.0110	2000	20	0.0110
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
2	Boscalid	0.04	125	12.5	0.0032	2700	27	0.0015
	Fluopicolide	0.04	155	15.5	0.0026	360	3.6	0.0111
	Zoxamide	0.04	4	0.4	0.1000	160	1.6	0.0250
3	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
4	Boscalid	0.02	125	12.5	0.0016	2700	27	0.0007
	Cyflufenamid	0.02	24	2.4	0.0083	1040	10.4	0.0019
	Fludioxonil	0.19	40	4	0.0475	230	2.3	0.0826
	Fluopyram	0.17	135	13.5	0.0126	42900	429	0.0004
	Iprovalicarb	0.12	9890	989	0.0001	22700	227	0.0005
	Myclobutanil	0.06	200	20	0.0030	2000	20	0.0030
	Penconazole	0.18	360	36	0.0050	1130	11.3	0.0159
	Pyrimethanil	0.04	1600	160	0.0003	10560	105.6	0.0004
	Tebuconazole	0.08	10	1	0.0800	4400	44	0.0018
	Chlorpyrifos-methyl	0.02	5	0.5	0.0400	410	4.1	0.0049
	Dimethoate	0.08	400	40	0.0020	30200	302	0.0003
5	Famoxadone	0.05	1.4	0.14	0.3571	11	0.11	0.4545
	Fludioxonil	0.06	40	4	0.0150	230	2.3	0.0261
	Iprovalicarb	0.11	9890	989	0.0001	22700	227	0.0005
	Myclobutanil	0.03	200	20	0.0015	2000	20	0.0015
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
Pond E								
1	Azoxystrobin	0.07	147	14.7	0.0048	470	4.7	0.0149
	Boscalid	0.27	125	12.5	0.0216	2700	27	0.0100
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Fluopicolide	0.04	155	15.5	0.0026	360	3.6	0.0111
	Fluopyram	0.21	135	13.5	0.0156	42900	429	0.0005
	Iprovalicarb	0.12	9890	989	0.0001	22700	227	0.0005
	Metrafenone	0.21	228	22.8	0.0092	820	8.2	0.0256

	Tetraconazol	0.02	300	30	0.0007	4400	44	0.0005
2	Boscalid	0.02	125	12.5	0.0016	2700	27	0.0007
	Dimethomorph	0.1	56	5.6	0.0179	6100	61	0.0016
	Folpet	4.53	8.1	0.81	**5.5926**	680	6.8	0.6662
	Fluopicolide	0.04	155	15.5	0.0026	360	3.6	0.0111
	Fluopyram	0.13	135	13.5	0.0096	42900	429	0.0003
	Iprovalicarb	3.05	9890	989	0.0031	22700	227	0.0134
	Kresoxim-methyl	0.22	13	1.3	0.1692	190	1.9	0.1158
	Metrafenone	0.05	228	22.8	0.0022	820	8.2	0.0061
	Myclobutanil	0.73	200	20	0.0365	2000	20	0.0365
	Penconazole	0.03	360	36	0.0008	1130	11.3	0.0027
	Pyrimethanil	0.03	1600	160	0.0002	10560	105.6	0.0003
	Zoxamide	0.14	4	0.4	0.3500	160	1.6	0.0875
	Dimethoate	0.06	400	40	0.0015	30200	302	0.0002
3	Folpet	0.58	8.1	0.81	0.7160	680	6.8	0.0853
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Fluopyram	0.20	135	13.5	0.0148	42900	429	0.0005
	Dimethoate	0.02	400	40	0.0005	30200	302	0.0001
4	Boscalid	0.15	125	12.5	0.0120	2700	27	0.0056
	Folpet	0.20	8.1	0.81	0.2469	680	6.8	0.0294
	Fludioxonil	0.22	40	4	0.0550	230	2.3	0.0957
	Fluopyram	0.18	135	13.5	0.0133	42900	429	0.0004
	Iprovalicarb	0.11	9890	989	0.0001	22700	227	0.0005
	Myclobutanil	0.03	200	20	0.0015	2000	20	0.0015
	Chlorpyrifos-methyl	0.02	5	0.5	0.0400	410	4.1	0.0049
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
5	Boscalid	0.26	125	12.5	0.0208	2700	27	0.0096
	Famoxadone	0.15	1.4	0.14	**1.0714**	11	0.11	**1.3636**
	Folpet	0.73	8.1	0.81	0.9012	680	6.8	0.1074
	Fludioxonil	0.25	40	4	0.0625	230	2.3	0.1087
	Fluopicolide	0.53	155	15.5	0.0342	360	3.6	0.1472
	Fluopyram	0.23	135	13.5	0.0170	42900	429	0.0005
	Iprovalicarb	0.36	9890	989	0.0004	22700	227	0.0016
	Metalaxyl M	0.24	9100	910	0.0003	27000	270	0.0009
	Metrafenone	0.3	228	22.8	0.0132	820	8.2	0.0366
	Myclobutanil	0.24	200	20	0.0120	2000	20	0.0120
	Tebuconazole	0.05	10	1	0.0500	4400	44	0.0011
	Zoxamide	0.02	4	0.4	0.0500	160	1.6	0.0125
	Chlorpyrifos-methyl	0.02	5	0.5	0.0400	410	4.1	0.0049

Table S3: Results of the Kendall-Theil Sen Siegel regression model to identify whether the pesticide contamination of ponds (sum of toxic units, STU) affects the investigated reproduction endpoints.

	Coefficient	**Estimate**	**df**	***p***
Fecundity	STU	13.52	50	< 0.001
Fertilization rate	STU	-0.24	46	< 0.001
Offspring survival	STU	-3.93	46	< 0.001
Offspring fitness	STU	-0.04	48	< 0.001

Table S4: Determined median, mean and standard deviation for the investigated reproductive endpoints and ponds.

Endpoint	Pond	Median	Mean	Standard deviation
Fecundity (eggs/g)	A	46	49	11
	B	55	54	11
	C	68	70	11
	D	71	69	23
	E	81	74	30
Fertilization rate (%)	A	99.2	98.9	0.9
	B	97.8	98.96	3.6
	C	99.7	100.0	0.5
	D	94.5	96.1	7.1
	E	92.0	94.4	8.5
Offspring survival (%)	A	97.0	98.9	4.1
	B	92.9	93.3	4.8
	C	94.7	95.0	4.2
	D	88.9	92.1	8.9
	E	54.2	66.3	36.0
Tadpole length (mm)	A	12.5	12.4	0.5
	B	11.8	11.8	0.5
	C	11.4	11.3	0.4
	D	11.8	11.7	0.8
	E	11.3	11.2	0.4

Chapter 5

Influence of forest and agricultural landscapes on biometry, age and genetic structure in Palmate Newt (*Lissotriton helveticus*) populations

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Abstract

Ponds in agricultural landscapes are often used by amphibians as breeding habitat. However, the characteristics of agricultural ponds and especially the surrounding area are usually said to be suboptimal for many amphibian species. Using suboptimal habitats might allow a species' survival and reproduction, but can have negative consequences at the individual and population level. In the present study, we investigated Palmate Newt (*Lissotriton helveticus*) populations from an intensive wine-growing region in southern Germany and compared them with populations located in a nearby forested area in terms of biometric traits, age and genetic structure. By analyzing over 900 adult newts from 11 ponds, we could show that newts reproducing in forest ponds were larger than newts reproducing in agricultural ponds. We did not find differences in the newt age and growth rate between habitat types. Therefore, differences in the body size of newts might already existed in larvae and/or juveniles, what might be related to a lower habitat quality for larvae and/or juveniles in the agricultural landscape. Body mass, body condition and sexual dimorphic traits (length of the caudal filament and max. height of the tail) correlated with body size, but no additional effect of the habitat type was found. The analysis of microsatellites revealed a higher genetic diversity in forest ponds. However, no clear sign of inbreeding was observed in any agricultural population, suggesting some degree of gene flow between them. We conclude, that agricultural ponds can be suitable habitats for the Palmate Newt and that conservation effort should aim to preserve them. The observed effects on body size indicate the need to increase the quality of the aquatic and terrestrial habitat for early life stages of this newt species in agricultural landscapes.

Keywords

Amphibia, Caudata, body size, carry-over effect, microsatellites, sexual dimorphic traits, skeletochronology

Introduction

Agriculture is the dominant land-use in large parts of Europe (Eurostat 2020), whereby increasing industrialization and therefore intensification could be observed over the last decades. Intensive agriculture is named as one of the main drivers of the current biodiversity crisis (Dudley & Alexander 2017, Marques et al. 2019). Still, some landscape structures within the agricultural landscape can serve as habitat for several species. One example is constructed ponds (e.g. for rain retention) which can be breeding habitats of amphibians (Knutson et al. 2004, Lenhardt et al. 2013, Rannap et al. 2020), one of the most endangered taxonomic groups of vertebrates (IUCN 2021). Most temperate amphibians rely not only on an appropriate aquatic, but also on a suitable terrestrial habitat where they spend most of their lifetime. Thus, amphibians reproducing in agricultural ponds can often be found directly within agricultural fields during their post-breeding migration (Kovar et al. 2009, Gert Berger et al. 2013, Salazar et al. 2016, Leeb et al. 2020a).

Both, the aquatic and terrestrial amphibian habitat, are expected to have a lower quality in intensive agricultures (i.e. crops like wheat or vine) compared to more natural areas. As intensive agriculture is linked to high pesticide and fertilizer use most agricultural soils are contaminated with agrochemicals (Hvězdová et al. 2018, Silva et al. 2019). Due to spray-drift (Crossland et al. 1982), run-off (Edwards et al. 1980), and drainages (Brown & van Beinum 2009) pesticides also contaminate the aquatic habitat of amphibians (Bókony et al. 2018, Adams et al. 2021). Pesticides and fertilizer can have negative effects on amphibians (Mann et al. 2009, Brühl et al. 2011). Besides these also other agricultural management practices like mowing of grass or mechanical tillage, can be an additional threat for amphibians (Pfeffer et al. 2011, Schuler et al. 2013, Leeb et al. 2020a). Further, the trophic resources for amphibians might be reduced in intensive agriculture, a circumstance known for birds (Wilson et al. 1999, Benton et al. 2002) or bats (Wickramasinghe et al. 2004, Put et al. 2018). Additionally, intensive agriculture often consists of low-structured monoculture what might not only indicates the absence of important structures like hiding places or hibernation sites, but also contributes to the fragmentation of the landscape and isolation of non-crop habitats (Landis 2017).

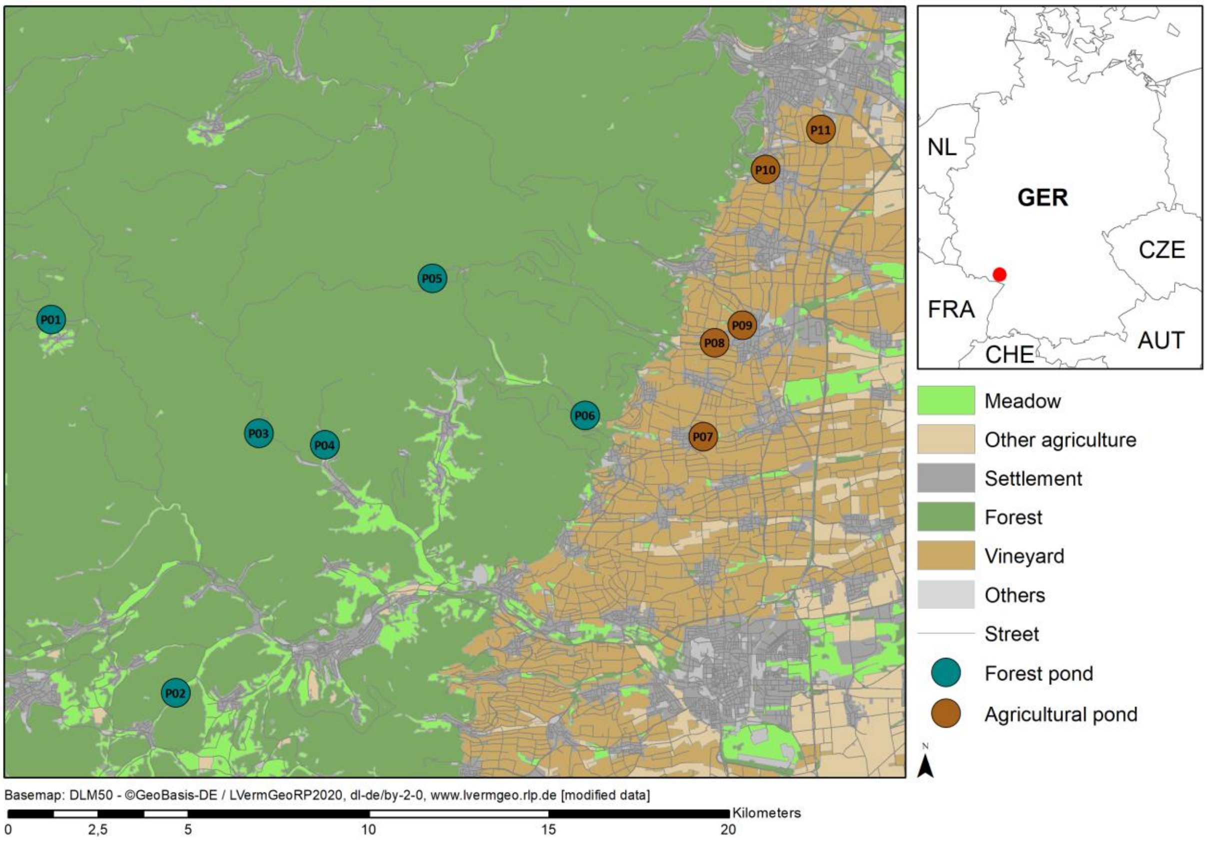
A low habitat quality can have negative consequences at the individual level, including decreased body mass of larvae and juveniles (Gray & Smith. 2005, Bókony et al. 2018), reduced body condition of adults (Brodeur et al. 2011) or reduced reproduction success (Adams et al. 2021). As a result, also effects at the population level (such as demographic structure; Bionda et al. 2018) or meta-population level (such as reduced gene flow between populations in agricultures; Lenhardt et al. 2017, Costanzi et al. 2018) are possible. However, in highly altered landscapes like agricultures, ponds can be regarded as stepping stone habitats between more favourable areas, as shown for example for dragonflies (Simaika et al. 2016). In some cases these artificial ponds are even considered of having the same value for amphibians as more natural habitats (Orchard et al. 2019). The importance of remaining agricultural ponds is enhanced in light of the observed pond loss during the last decades. For example, 57% of ponds disappeared between 1975 and 2006 in an agricultural landscape in northern France (Curado et al. 2011). Thus, there is an open debate on the role of agricultural ponds for amphibian populations. Additional investigations on how intensive agriculture might shape amphibian populations are vital for conservation measures.

In the present study, we assessed the value of agricultural ponds for amphibians by focusing on populations of the Palmate Newt, *Lissotriton helveticus* (Razoumowsky, 1789) from an intensive wine-growing region in southwestern Germany. We compared biometric traits as well as demographic and genetic population structure between populations from ponds in the agricultural landscape and the forest. The Palmate Newt is a small semiaquatic newt that is common in western Europe (Least Concern by the IUCN (Arntzen et al. 2009) and the red list Germany (Schlüpmann & Grosse 2020)). Although it can be found in a variety of habitats, including agricultural landscapes (Secondi et al. 2007, Lenhardt et al. 2013, Trochet et al. 2016), its presence is often linked to forests (Denoël & Lehmann 2006, Schlüpmann 2006, Manenti et al. 2013). Johanet et al. (2009) even showed a positive correlation between body size of Palmate Newts and forest cover in the surroundings of a pond. Assuming that in our study area forests represent better habitats for *L. helveticus*, we expect newts reproducing in forest ponds (hereafter "forest newts") to be larger, to have a higher body condition and more pronounced sexual dimorphic traits than newts reproducing in agricultural ponds (hereafter "agriculture newts"). Additionally, we hypothesize that agriculture newts have a lower annual survival and thus a shifted demographic structure. Finally, we expect that these morphological and demographic effects would, together with an assumed reduced gene flow between populations in the agricultural landscape (Lenhardt et al. 2017), be reflected in the genetic structure of the populations, with populations in forest ponds having a higher genetic diversity and lower degree of inbreeding.

Material and methods

Study area and pond characterization

The study was conducted between Landau in der Pfalz and Neustadt an der Weinstraße (Rhineland-Palatinate, Germany) in an area of about 20 × 16 km. The eastern part of the study area is dominated by vineyards, while the Palatine forest (part of the Palatinate Forest-North Vosges Biosphere Reserve) is located in the western part (Fig. 1). In both parts several ponds are located for which the occurring amphibian species had been mapped during the last years (see e.g. Lenhardt et al. 2013). For the present study we selected 11 ponds inhabited by *L. helveticus* and, based on their location, classified them a priori (pond type) as forest (n = 6) or agricultural pond (n = 5; Fig. 1). Most agricultural ponds were constructed for rain retention and are within or next to vineyards. To characterize each pond, we measured pH and conductivity (µS/cm; both water parameters measured with the multi-parameter instrument Multi 340i, WTWW, Germany) and visually estimated the percentage of the pond's area covered with submersed vegetation in April 2018. We further analysed the land use in a radius of 400 m around each pond, a distance that is within the migration capacity of *L. helveticus* (Joly et al. 2001) and that has been used in comparable studies about this species (Johanet et al. 2009). Based on a vector landscape model of Rhineland-Palatinate (ATKIS DLM50) the percentage of forests (including groves), agriculture (without meadows), meadows and settlements (including industrial areas) as well as the length of the street network (road with solid surface) was calculated around each pond.



Basemap: DLM50 - ©GeoBasis-DE / LVermGeoRP2020, dl-de/by-2-0, www.lvermgeo.rlp.de [modified data]

Fig. 1: Map of study area with all sampled forest (n = 6) and agricultural ponds (n = 5).

Newt sampling and biometric measurements

Between 26 March and 13 April 2018 all ponds were visited three times to capture newts with dip nets for biometric analysis. The average time between two sampling events of the same pond was 6.2 days. Three ponds were also sampled at the end of May to assess if the phenology of newts (e.g. caused by different microclimatic conditions at a pond) influences sexual dimorphic traits. Adult newts were captured and were transported to a facility of the University Koblenz-Landau (average distance to the ponds = 9.6 km). Newts were kept in groups of up to four individuals in 20 L aquaria filled with tap water for about 24 hours to allow defecation and thus to minimize the influence of recently consumed prey on the measurements. During this time, newts were not fed. Newts were sexed, weighed to the nearest 0.001 g (CP153 analytical balances, Sartorius, Germany) and lateral and dorsal photos were taken in small photo-aquaria. Based on a reference scale on each photo the snout–vent length (SVL) was measured in ImageJ (Schneider et al. 2012). SVL and the body mass were used to calculate the scaled mass index (SMI; Peig & Green 2009) as indicator of body condition separately for males and females. For males, the length of the caudal filament (CF) and the maximum height of the tail (T) were measured. Biometric data from all three samplings were pooled, because there were only a few days between two samplings of the same pond and we expected biometric changes to be negligible. Newts from the first and second sampling event were marked by clipping of the longest finger of the left front limp with a sharp surgical scissor to avoid multiple sampling of individuals. Finger clips were stored in 70% alcohol at -20°C and used for age determination (bones) and genetic analysis (tissue). At latest 48 h after capturing, newts were released at their capture site. The handling of Palmate Newts was approved by the ”Struktur- und Genehmigungsdirektion Süd Referat 42 – Obere Naturschutzbehörde” (Neustadt an der Weinstraße, Germany; approval numbers: 42/553-254 – 456/16, 42/553-252/ 456(17) and 42/553-254/ 456-18).

Genetic analysis

For genetic analysis we used tissue (finger clips) taken between 2016 and 2018. Genomic DNA was extracted using the high salt DNA extraction method (Aljanabi 1997). Nine microsatellite loci (Drechsler et al. 2013) were amplified in two multiplexed polymerase chain reactions (PCR 1: loci Lh7, Lh13, Lh14, Lh44 and Us9; PCR 2: Lh9, Lh16, Lh17 and Lh19) using the Type-it Microsatellite PCR Kit (QIAGEN). Fluorescence labelled PCR products were measured in a CEQ 8000 Sequencer (Beckman Coulter) and peaks were scored using GeneMarker V1.95 (SoftGenetics). During scoring we noticed that there is a mistake in Drechsler et al. (2013) as the described primer sequences of Lh9 and Lh17 are identical and are consequently amplifying the same region in the genome. Thus, we combined the scoring results of Lh9 and Lh17 (in the following locus Lh9/17). Checking the microsatellite data for null-alleles and scoring errors with Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) revealed the presence of null alleles in several populations. As null alleles can bias standard population genetic parameters (Chapuis & Estoup 2007), we excluded the loci Lh13, Lh14 and Lh9/17 from our genetic analysis, reducing the effective number of loci to five. We used GENEPOP 4.6 (Rousset 2008; implemented in the R-package “genepop”) to test for linkage disequilibrium between primer pairs of loci over all populations. For each population, the observed and expected heterozygosity (H_o and H_e), the allelic richness (AR; calculated using 1,000 re-samples) and the inbreeding coefficient (F_{IS}) with its 95% confidence interval (1,000 bootstrap iterations) were calculated with the function “divBasic” in the R-package “diveRsity” (Keenan et al. 2013). The function “divBasic” was also used to test each population and Loci for Hardy-Weinberg equilibrium (HWE).

Age determination

For age determination fingers from newts of two forest and two agricultural ponds were used (Table 1). Skeletochronological age determination followed standard laboratory protocols (Sinsch 2015). Samples were embedded in Historesin^{TM} (JUNG) and stained with 0.5% cresylviolet (Sinsch et al. 2018). The midsection of the bone (diaphysis) was cross-sectioned at 12 µm using a JUNG RM2055 rotation microtome. Cross sections were examined under a light microscope (OLYMPUS BX 50) for the presence of growth marks at magnifications of 400x. We distinguished strongly stained lines of arrested growth (LAGs) in the periosteal bone, separated by faintly stained broad growth zones

(Sinsch et al. 2007). We selected diaphysis sections in which the size of the medullar cavity was at its minimum and that of periosteal bone at its maximum. The number of LAGs was assessed independently by two authors (FB, US) to estimate age.

Table 1: Number of Palmate Newts per pond used in the study. The effective number of individuals used in the analysis is lower as some parameters could not be measured. Age determination failed in eight individuals.

		Number individuals used for					
Pond-ID	**Classification**	**genetic analysis**	**biometric analysis**			**skeletochronology**	
			Males	**Females**	**Males May**	**Males**	**Females**
P01	Forest	20	35	46	-	-	-
P02	Forest	18	49	31	-	-	-
P03	Forest	23	45	36	11	20	18
P04	Forest	19	30	36	-	-	-
P05	Forest	20	41	24	26	20	15
P06	Forest	18	26	23	-	-	-
P07	Agriculture	20	56	33	2	-	-
P08	Agriculture	22	13	12	-	-	-
P09	Agriculture	23	39	56	-	20	19
P10	Agriculture	18	78	72	-	-	-
P11	Agriculture	16	67	75	-	20	20
	Sum	217	479	444	39	80	72

Statistical analysis

To validate our a priori classification into forest and agricultural ponds (pond type), we performed a principal component analysis (PCA) with scaled variables describing the ponds (pH, conductivity, submersed vegetation) and their surroundings (land use in a radius of 400 m). Further, we used Mann-Whitney-U-tests to test in which variables forest and agricultural ponds differ.

Mixed models with pond-ID as random effect were used to assess the relation between pond type and biometric traits. As Mann-Whitney-U-tests revealed no difference between forest and agricultural ponds in percentage of meadows around and submersed vegetation in a pond (Table 2), both variables were used as fixed effects in all models to see if the hypothesized effect of pond type still exists. Sex of an individual was used as additional fixed effect to take sex specific differences in biometric traits into account. For newt size (SVL) this results in the model SVL ~ pond type + meadow + submersed vegetation + sex + (1|pond-ID).

A model averaging approach was used to identify significant variables (Burnham & Anderson 2002) describing the variation of SVL. For this purpose, a set of candidate models with all possible combinations of variables was generated based on the full model and the Akaike Information

Criterion with a correction for small sample sizes (AICc) was calculated. We considered candidate models with a ΔAICc < 4 to the best model to have empirical support (Burnham & Anderson 2002) and used them to calculate averaged model parameters, p-values and 95% confidence-intervals. We used this approach also with body mass, SMI and sexual dimorphic traits (CF and T) as response variable, including SVL and interaction of SVL and pond type as additional fixed effects (see Table 3 for full models). For CF and T we excluded sex and included SMI as additional variable in the models. To test if the age of an individual had an effect on the model responses, we calculated all models again including age as fixed effect. Because age was only determined for four ponds, these models included only a subset of our data. In general, linear mixed models (LMM) were used, but in cases where a Shapiro-Wilk test revealed not randomly distributed residuals of one of the candidate models, model averaging was based on a generalized linear mixed model (GLMM) with a Gaussian error distribution and a log-link function.

Mann-Whitney-U-tests were used to test if there are differences in the genetic parameters AR and F_{IS} between forest and agricultural ponds. To analyze if the growth rate (increase of SVL with the age in the adult stage) differs for males or females reproducing in forest or agricultural ponds, we used a LMM and tested the effect of the interaction of sex, pond type and age on SVL and used the pond-ID as random effect. To test for differences in the median age between males and females reproducing in forest and agricultural ponds, respectively, a Kruskal-Wallis test was used. To assess if the phenology of newts has an effect on the sexual dimorphic traits within a breeding season we compared CF and T from individuals captured in March/April with individuals captured in May in the same pond with a t- and a Mann-Whitney-U-test, respectively.

Variables of t-tests were tested for normality with Shapiro-Wilk tests and for variance homogeneity across tested groups with a Levene's test. All statistical analyses were carried out in R (version 3.4.3; R Development Core Team 2020). Mixed models were calculated with the function "lmer" (LMM) or "glmer" (GLMM) in the R-package "lme4" (Bates et al. 2015). The R-package "MuMIn" (Barton 2020) was used to generate sets of the full model (function "dredge") and to calculate averaged model parameters ("model.avg"). For the LMM to test for differences in the growth rate p-values were calculated with the Satterthwaite's method implemented in the package "lmerTest" (Kuznetsova et al. 2017). The criterion for significance was 0.05.

Table 2: Classification, location and characteristics of all ponds and information about the land use in the surroundings. Mann-Whitney-U-tests (U-Test) were used to test in which variables forest and agricultural ponds differ. P-values < 0.05 are presented in bold.

		Coordinates		**Pond parameters**				**Land use in 400 m**						
									Agriculture					
Pond-ID	**Classification**	**x**	**y**	**Altitude (m)**	**pH**	**Conductivity (µS/cm)**	**Submersed vegetation (%)**	**Forest (%)**	**Total (%)**	**Vineyard (%)**	**Settlement & Industry (%)**	**Meadow (%)**	**Other landuse (%)**	**Streets (m)**
P01	Forest	7.856941	49.284492	381	6.05	56	80	92	0	0	2	6	0	1826
P02	Forest	7.906574	49.191834	231	7.11	162	0	91	0	0	0	9	0	818
P03	Forest	7.936898	49.256871	264	6.46	59	40	100	0	0	0	0	0	594
P04	Forest	7.962192	49.254196	222	7.52	78	95	88	0	0	1	5	6	1139
P05	Forest	8.002318	49.296102	508	6.33	72	5	100	0	0	0	0	0	1012
P06	Forest	8.061345	49.262409	254	7.05	209	50	91	0	0	1	8	1	2590
P07	Agriculture	8.106360	49.257465	175	7.11	1049	95	0	66	66	26	4	5	6057
P08	Agriculture	8.110346	49.280868	191	7.56	215	5	7	82	82	8	0	3	5228
P09	Agriculture	8.120843	49.285363	179	7.9	790	85	0	35	35	61	2	2	5740
P10	Agriculture	8.128984	49.324232	195	7.42	271	60	5	48	48	46	0	0	5785
P11	Agriculture	8.149951	49.334327	138	7.46	372	70	0	95	95	1	0	3	3554
Median	Forest			259	6.76	75.0	45.0	91.6	0.0	0.0	0.3	5.5	0.0	1075.7
	Agriculture			179	7.46	372.0	70.0	0.5	65.6	65.6	25.9	0.0	3.3	5740.3
U-Test	W			30	3.5	0	10	30	0	not tested	1	23	not tested	0
	p			**0.004**	**0.004**	**0.004**	0.409	**0.008**	**0.004**		**0.013**	0.151		**0.004**

Results

Pond characterization and classification

The first principal component (PC1, explaining 57.6% of the total variance) of the PCA confirmed our a priori classification in forest and agricultural ponds as forest ponds had a negative and agricultural ponds a positive PC1 (Supplementary document 1; see Table 2 for pond characteristics). Also, Mann-Whitney-U-tests revealed differences between forest and agricultural ponds. Forest ponds had a significantly higher percentage of forests, a lower percentage of agricultural land use and settlements and a less dense network of streets in a distance of 400 m around a pond. They were situated at higher altitudes and pond water had a lower pH and a lower conductivity compared to agricultural ponds. The percentage of meadows around a pond and the submersed vegetation in a pond did not differ between forest and agricultural ponds.

Sampling and measured traits

A total of 923 individuals from six forest ponds (mean n = 70.3 ± 12.8) and five agricultural ponds (mean n = 100.2 ± 50.1) were captured and measured in March/April 2018 (Table 1). The effective number of individuals in our analysis was lower, as some photos did not allow an exact measurement of all parameters. Skeletochronology was used to estimate age from 152 individuals inhabiting four ponds (mean n = 38.0 ± 2.2). Age determination failed in eight individuals because the phalanges sampled were regenerates. Table 4 gives mean values, standard deviations and the ranges of biometric measurements (Fig. 2 for boxplots) and age structure (Fig 3. for age distribution and growth) of agriculture and forest newts. On average 19.7 ± 2.2 individuals per pond were used for genetic analysis. There was no evidence for linkage disequilibrium for any microsatellite loci (Supplementary document 6). Deviation from HWE was only detected for one locus in one population (Table 5, Supplementary document 7). Details about genetic parameters for each population are given in Table 5 (Fig. 4 for boxplots).

Modelling of traits

Model-averaged coefficients to describe the variation of SVL are based on a set of three candidate models with a ΔAICc < 4 (see Supplementary document 5 for a full list of all candidate models). Candidate models included all variables of the full model, but only pond type and sex of an individual had a significant effect on the SVL (Table 3), with individuals reproducing in forest ponds and females being larger. The body mass of an individual was dependent on the sex and SVL, but not pond type. Submersed vegetation had a negative effect on the body mass with a low estimate. Model-averaged coefficients of candidate models describing the variation of the length of the caudal filament (CF) and the maximum height of the tail (T) revealed a significant positive effect of SVL and SMI on both traits and no additional effect of pond type. Comparing CF and T of males captured in March/April with males captured in May showed different results for each investigated pond: In the pond P05 the CF was significant longer and T was significant higher in March, while in the pond P03 T was higher in May and no difference was observed in CF. For both traits, higher values were observed in P07 in March/April, but as only two individuals could be captured in May, no statistical analysis was possible (Supplementary document 4).

Including the age of an individual in the full model for the SVL confirmed effects of sex and pond type but showed also that older individuals are larger. Age also had an additional effect on SMI, but not on body mass, T or CF (Table 4). We could not detect a difference in the median age of males and females reproducing in forest and agricultural ponds, respectively (median age of each test group = 3; Kruskal-Wallis test: Df = 3, $\chi^2 = 2.830$, $p = 0.419$). As the interaction of sex, pond type and age had no significant effect on SVL ($F = 1.205$, $p = 0.274$), no difference in the growth rate was found (Fig. 3B). A Mann-Whitney-U-test revealed that newt populations from forest ponds had a significant higher AR than populations from agricultural ponds ($W = 27$, $p = 0.035$). No differences were found for F_{IS} ($W = 13$, $p = 0.792$) and 95% confidence intervals included zero for most populations.

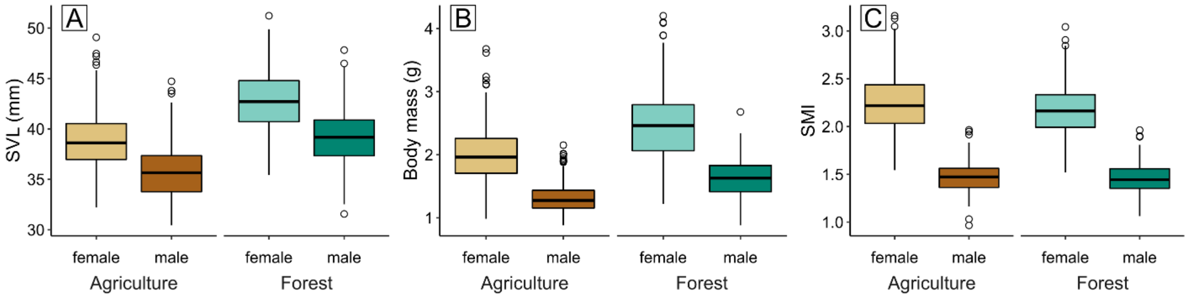


Fig. 2: Boxplots of the snout–vent length (SVL; A), the body mass (B) and the scaled mass index (SMI; C) of male and female *Lissotriton helveticus* reproducing in forest (n = 6) and agricultural (n = 5) ponds. In each boxplot, the boundaries of the box are the 25th and 75th percentiles and the whiskers correspondent to the lowest and largest value no further than 1.5 times from the 25th and 75th percentiles away. Data points beyond the whiskers are shown as unfilled circles. Mean values are given in Table 2. For effects of the pond type and the sex on the traits see Table 3.

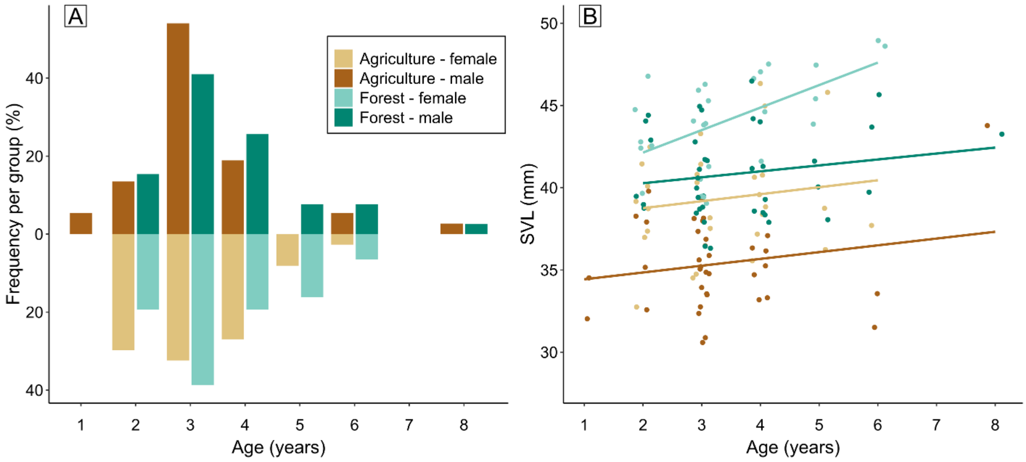


Fig. 3: Demographic structure of *Lissotriton helveticus* captured in forest and agricultural ponds (A) and correlation between snout-vent length (SVL) and age (B). No difference in the median age of males and females reproducing in forest and agricultural ponds, respectively, was found. The age had a significant effect on the SVL, but growth did not differ between males and females reproducing in forest and agricultural ponds.

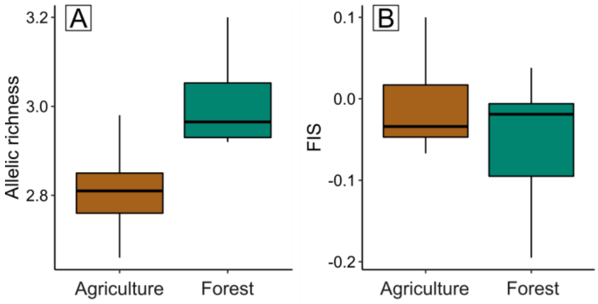


Fig. 4: Boxplots showing the allelic richness (A) and the inbreeding coefficient F_{IS} (B) of *Lissotriton helveticus* populations situated in the forest (n = 6) and the agriculture (n = 5). In each boxplot, the boundaries of the box are the 25^{th} and $75t^{h}$ percentiles and the whiskers correspondent to the lowest and largest value no further than 1.5 times from the 25^{th} and 75^{th} percentiles away. While a significant difference between forest and agricultural ponds was found for the allelic richness, no difference was found for F_{IS}

Table 3: Results of model averaging to describe the response variables (SVL = snout–vent length; SMI = scaled mass index; T = max. height of Tail; CF = caudal filament) with and without the age of an individual as additional fixed effect with the estimate, standard error (SE) and 95% confidence interval. Model averaging was based on a set of candidate models with a ΔAICc < 4 (see Supplementary document 5 for all candidate models). GLMM = generalized linear mixed model with an Gaussian error distribution and a log-link function; LMM = linear mixed model; : = interaction between fixed effects; (1|Pond-ID) = random effect. P-values < 0.05 are presented in bold.

Response	**Variable**	**Estimate**	**SE**	**lower CI 95%**	**upper CI 95%**	**z**	**p**
Full model: GLMM (SVL ~ Sex + Pond type + Submersed vegetation + Meadow + (1|Pond-ID))							
SVL	Intercept	3.620	0.021	3.579	3.662	170.125	**< 0.001**
(n = 856)	Sex - female	0.082	0.004	0.074	0.091	18.467	**< 0.001**
	Pond type - forest	0.101	0.022	0.057	0.145	4.491	**< 0.001**
	Submersed vegetation	0.000	0.000	-0.001	0.000	1.381	0.167
	Meadow	-0.006	0.004	-0.013	0.001	1.593	0.111
Full model incl. age: LMM (SVL ~ Sex + Age + Pond type + Submersed vegetation + Meadow + (1|Pond-ID))							
SVL	Intercept	34.081	1.839	30.456	37.707	18.425	**< 0.001**
(n = 132)	Sex - female	3.668	0.476	2.727	4.610	7.635	**< 0.001**
	Age	0.557	0.192	0.177	0.937	2.873	**0.004**
	Pond type - forest	4.778	1.756	1.311	8.245	2.701	**0.007**
	Submersed vegetation	-0.004	0.016	-0.035	0.027	0.269	0.788
	Meadow	-0.099	0.960	-1.998	1.801	0.102	0.919
Full model: GLMM (Body mass ~ Sex + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID))							
Body mass	Intercept	-1.547	0.091	-1.726	-1.368	16.933	**< 0.001**
(n = 855)	Sex - female	0.256	0.009	0.237	0.274	27.502	**< 0.001**
	SVL	0.052	0.002	0.048	0.056	25.847	**< 0.001**
	Pond type - forest	0.141	0.133	-0.120	0.402	1.058	0.290
	Pond type * SVL	-0.003	0.003	-0.009	0.003	0.937	0.349
	Submersed vegetation	-0.001	0.000	-0.002	-0.000	2.053	**0.040**
	Meadow	-0.001	0.003	-0.005	0.004	0.201	0.841
Full model incl. age: GLMM (Body mass ~ Sex + Age + Pond type + SVL + Pond type : SVL + Submersed vegetatio + Meadow + (1|Pond-ID))							
Body mass	Intercept	-1.120	0.143	-1.403	-0.838	7.778	**< 0.001**
(n = 132)	Sex - female	0.302	0.023	0.255	0.348	12.714	**< 0.001**
	Age	0.004	0.007	-0.010	0.018	0.576	0.565
	SVL	0.042	0.003	0.036	0.049	12.749	**< 0.001**
	Pond type - forest	0.004	0.035	-0.065	0.073	0.113	0.910
	Submersed vegetation	-0.002	0.001	-0.003	-0.000	2.061	**0.039**
	Meadow	-0.028	0.029	-0.085	0.030	0.947	0.344
Full model: GLMM (SMI ~ Sex + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID))							
SMI	Intercept	0.997	0.067	0.865	1.128	14.820	**< 0.001**
(n = 855)	Sex - female	0.467	0.009	0.449	0.485	51.893	**< 0.001**
	SVL	-0.016	0.002	-0.019	-0.013	10.227	**< 0.001**
	Pond type - forest	0.025	0.050	-0.072	0.122	0.502	0.616
	Pond type * SVL	0.000	0.001	-0.002	0.002	0.061	0.951
	Submersed vegetation	-0.001	0.000	-0.002	-0.000	1.688	0.091
	Meadow	-0.001	0.003	-0.008	0.005	0.355	0.723
Full model incl. age: GLMM (SMI ~ Sex + Age + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID))							

SMI	Intercept	1.497	0.145	1.210	1.784	10.229	**< 0.001**
(n = 132)	Sex - female	0.526	0.024	0.478	0.574	21.645	**< 0.001**
	Age	0.020	0.010	0.001	0.040	2.056	**0.040**
	SVL	-0.028	0.003	-0.035	-0.021	8.131	**< 0.001**
	Pond type - forest	-0.029	0.097	-0.220	0.161	0.303	0.762
	Pond type * SVL	0.001	0.002	-0.004	0.005	0.230	0.818
	Submersed vegetation	-0.002	0.001	-0.004	0.000	2.072	**0.038**
	Meadow	-0.057	0.032	-0.121	0.006	1.774	0.076
Full model: LMM (T ~ Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID))							
T	Intercept	-0.653	0.894	-2.410	1.103	0.729	0.466
(n = 472)	SVL	0.159	0.017	0.125	0.192	9.183	**< 0.001**
	SMI	2.280	0.283	1.724	2.837	8.035	**< 0.001**
	Pond type - forest	-0.102	0.233	-0.560	0.356	0.437	0.662
Full model incl. age: LMM (T ~ Age + Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID))							
T	Intercept	-3.031	2.705	-8.429	2.367	1.101	0.271
(n = 74)	SVL	0.197	0.052	0.093	0.301	3.710	**< 0.001**
	SMI	3.059	0.695	1.673	4.446	4.324	**< 0.001**
	Pond type - forest	-0.493	0.989	-2.459	1.474	0.491	0.623
	Meadow	0.037	0.604	-1.168	1.243	0.061	0.952
Full model: LMM (CF ~ Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID))							
CF	Intercept	-3.597	1.376	-6.300	-0.894	2.608	**0.009**
(n = 468)	SVL	0.138	0.024	0.091	0.184	5.770	**< 0.001**
	SMI	1.647	0.368	0.923	2.371	4.459	**< 0.001**
	Pond type - forest	-0.320	0.899	-2.084	1.445	0.355	0.723
	Pond type : SVL	-0.004	0.019	-0.042	0.034	0.209	0.834
	Submersed vegetation	0.005	0.010	-0.014	0.023	0.492	0.622
Full model incl. age: LMM (CF ~ Age + Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID))							
CF	Intercept	-0.453	4.933	-10.166	9.259	0.091	0.927
(n = 75)	SVL	0.107	0.096	-0.082	0.296	1.110	0.267
	SMI	0.845	1.018	-1.166	2.855	0.823	0.410
	Pond type - forest	-1.228	1.506	-4.213	1.758	0.806	0.420
	Submersed vegetation	0.001	0.009	-0.015	0.018	0.163	0.870
	Meadow	-0.313	0.900	-2.101	1.475	0.343	0.732

Table 4: Mean, standard deviation (SD) and range of the snout–vent length (SVL), body mass, scaled mass index (SMI), caudal filament (CF), max. height of the tail (T) and age of males and females reproducing in forest and agricultural ponds (see Figs 2 + 3). Additionally, the allelic richness (AR) and the inbreeding coefficient (F_{IS}) for agricultural and forest populations are given (see Fig. 4).

		Agricultural pond		**Forest pond**	
		Male	**Female**	**Male**	**Female**
SVL (mm)	Mean	35.8	38.9	39.6	43.0
	SD	2.7	2.9	2.7	2.9
	Range	30.4 - 44.7	32.2 - 49.1	33.3 - 47.8	35.4 - 51.2
Body mass (g)	Mean	1.316	2.0	1.677	2.541
	SD	0.238	0.4	0.279	0.522
	Range	0.881 - 2.152	0.985 - 3.677	1.029 - 2.678	1.565 - 4.202
SMI	Mean	1.5	2.2	1.5	2.2
	SD	0.2	0.3	0.2	0.3
	Range	1.0 - 2.0	1.5 - 3.2	1.1 - 2.0	1.5 - 3.0
CF (mm)	Mean	4.5	-	3.8	-
	SD	1.4	-	1.4	-
	Range	1.0 - 8.2	-	0.6 - 8.0	-
T (mm)	Mean	8.6	-	8.8	-
	SD	1.0	-	1.0	-
	Range	6.2 - 11.9	-	6.6 - 11.5	-
Age (Years)	Mean	3.2	3.2	3.6	3.5
	SD	1.3	1.1	1.3	1.2
	Range	1 - 8	2 - 6	2 - 8	2 - 6
AR	Mean	2.810		3.008	
	SD	0.118		0.110	
	Range	2.660 - 2.980		2.920 - 3.200	
F_{IS}	Mean	-0.006		-0.053	
	SD	0.067		0.087	
	Range	-0.067 - 0.100		-0.195 - 0.038	

Table 5: Genetic parameters of each population with the allelic richness (AR), the expected and observed heterozygosity (H_e and H_o), the inbreeding coefficient (FI_S) with its lower (F_{IS} low) and upper (F_{IS} high) 95% confidence intervals and the p-value from a Chi-square test for goodness-of-fit to Hardy-Weinberg equilibrium (p-HWE) are given.

Pond-ID	**Classification**	**AR**	**H_e**	**H_O**	**F_{IS}**	**F_{IS} low**	**F_{IS} high**	**p-HWE**
P01	Forest	3.20	0.47	0.53	-0.118	-0.243	0.015	0.854
P02	Forest	3.07	0.49	0.50	-0.012	-0.171	0.151	0.784
P03	Forest	3.00	0.47	0.48	-0.026	-0.182	0.128	0.841
P04	Forest	2.93	0.44	0.42	0.038	-0.142	0.189	0.075
P05	Forest	2.92	0.44	0.52	-0.195	-0.334	-0.064	0.854
P06	Forest	2.93	0.44	0.44	-0.004	-0.181	0.180	0.597
P07	Agriculture	2.66	0.38	0.41	-0.067	-0.242	0.123	0.760
P08	Agriculture	2.85	0.45	0.41	0.100	-0.107	0.301	0.100
P09	Agriculture	2.98	0.38	0.38	0.017	-0.102	0.142	0.552
P10	Agriculture	2.81	0.39	0.40	-0.034	-0.230	0.159	0.775
P11	Agriculture	2.76	0.49	0.51	-0.047	-0.191	0.099	0.649

Discussion

Agricultural ponds are often used as breeding habitat by amphibians. Understanding if intensive agriculture shapes biometric traits as well as the demographic and genetic structure of amphibian populations inhabiting these ponds helps to assess the value of these ponds for conservation management. As the presence of Palmate Newts (*L. helveticus*) is often linked to forest habitats, we hypothesized that agricultural landscape may represent low quality habitats. By analysing over 900 newts from 11 ponds we found evidence that this hypothesis holds correct in some aspects, but also that agricultural ponds can be suitable breeding habitats for Palmate Newts.

Biometric traits

In our study, Palmate Newts captured in agricultural ponds were smaller than those captured in forest ponds, which was true even if sex and age of an individual were considered. In newts, a larger body size can be advantageous. For example, a larger body size of females can result in more and larger eggs (Nobili & Accordi 1997, Verrell & Francillon 2009) and a more pronounced parental care (Tóth et al. 2011) in the closely related smooth newt (*L. vulgaris*). The positive effect of forests is in line with Johanet et al. (2009), where a correlation between forest cover and body size was found for male and female Palmate Newts in Western France. Also Secondi et al. (2007) found a trend between body size and forest cover for males in the same study area. Trochet et al. (2016) showed that the length of the hindlimbs of Palmate Newts in Southern France was correlated with the distance to the closest forest. Although the SVL of an individual had a significant effect on the length of the hindlimbs, no significant correlation between the SVL and other environmental parameters was found. Results from a laboratory study indicate that the exposure to nitrate, which is used as fertilizer and thus can often be found in agricultural ponds, can affect the body size of males (Secondi et al. 2009). Although fertilizer are hardly used in viticulture, contaminations could be one explanation for the smaller body sizes we found in agriculture newts in our study.

The body size of newts in our agricultural landscape (males: 35.8 mm; females: 38.9 mm) is still within the body size range of other European populations. Arntzen et al. (1998) reported males with a mean SVL of 34 mm in Mayenne (western France) and Denoël et al. (2019) a mean SVL of 38.9 mm of males and females from Larzac (France). In northeast Andorra males and females with a mean SVL of 36.0 mm and 38.8 mm, respectively, were found in two Pyrenean Lakes at an altitude of 2,300 m a.s.l. (Amat et al. 2010). One might assume that the high elevation and thus low temperatures and short activity periods might limit the growth of *L. helveticus* in the Pyrenees, but in fact, there is a general trend for an increase in body size with elevation in many amphibian species (Morrison & Hero 2003). Although the elevation gradient in our study is low (138–508 m; Table 2), the higher location of forest ponds (mean = 310 m; mean agricultural ponds = 176 m) might contribute to the observed differences in SVL. Interestingly, we found a negative effect of body size on body condition, which could indicate that it is harder for large newts to cover their food demand. Differences in body mass and body condition between forest and agriculture newts were a result of differences in the SVL and the habitat type had no additional effect on these traits.

Sexual dimorphic traits

In Palmate Newts sexual dimorphic traits play an important role in female mate choice (Cornuau et al. 2012, 2014). They can be even more important than body size, as they directly reflect the current fitness of an individual and not unfavourable conditions during earlier life stages (Haerty et al. 2007). As we assumed forest ponds to have a higher habitat quality and thus to allow a higher fitness of newts, we expected that forest newts have a longer caudal filament and a higher tail. However, pond type had no significant effect on both traits, and differences are only caused by a correlation with SVL and SMI. By comparing the sexual dimorphic traits measured in March/April and May we found that they are highly dependent on the time of measurement, but also on the pond. In Palmate Newts, sexual dimorphic traits are developed in the water and are regressed when they leave the aquatic phase at the end of the reproductive period (Griffiths & Mylotte 1988), whereby the exact time depends on microclimatic conditions. Consequently, we detected in the pond at the highest altitude (P05), with a presumed rougher microclimate and later migration of the newts to the pond, less pronounced sexual dimorphic traits in March/April than in May. In contrast, most individuals already left the agricultural pond P07 in May, so we were only able to catch two males with hardly any sexual dimorphic traits left. Incomplete development of the sexual traits in March/April would also explain the correlation with the SVL, which is in contrast to Cornuau et al. (2012) and Haerty et al. (2007). The significant correlation of the sexual dimorphic traits and the SMI is in line with Cornuau et al. (2014) and confirms their general suitability as fitness indicators. However, the dependence on time of measurement and pond questions the ability of our models to answer the question if forest newts have a higher fitness. This raises general concerns about the reliability of both traits as indicator of fitness in field studies where ponds at different altitudes and/or microclimatic conditions are compared.

Demographic traits

Differences in the demographic structure with older individuals in forest ponds would explain the differences in body size between forest and agricultural ponds. However, the skeletochronology revealed no difference in the median age between habitat types. Thus, it can also be assumed that there is, at least in adults, no increased mortality in the agricultural populations in our study area. Orchard et al. (2019) did not find differences in the demographic structure between crested newt populations (*Triturus cristatus*) from agricultural ponds and ponds from favourably managed sites, and concluded that agricultural ponds can harbour sustainable crested newt populations. While Amat et al. (2010) and Miaud (1991) reported *L. helveticus* with an age ranging from 3–9 (median = 5 years) and 4–8 years, respectively, the age of individuals from our study ranged between 1 and 8 years with a median of 3 years. Thus, a general shift in the demographic structure towards younger age groups can be observed in our study compared to Palmate Newts from Andorra and France, indicating favorable environmental conditions both in forest and agricultural habitats.

Carry-over effects and selection for small individuals

We could show a correlation between body size and age, but did not find differences in the growth rate (i.e. increase of SVL with the age in the adult stage) between males and females reproducing in agricultural and forest ponds. Thus, differences in the body size might be related to different conditions in the larval or juvenile phase that are transferred to the adult life stage (i.e. carry-over effects). Jennette et al. (2019) got similar results when comparing American toads (*Anaxyrus americanus*) and wood frogs (*Lithobates sylvaticus*) from rural and urban landscapes in Maryland (USA) and explained it with similar habitat qualities for adults, but lower quality of larval or juvenile habitats in urban areas. In our study area, larvae or juveniles might face a decreased food quality/quantity in agricultural ponds and landscapes, which could be compensated by adults for example by having a wider prey spectrum. Nobili & Accordi (1997) explained differences in the body size of larvae and adults between different smooth newt populations as a consequence of different water stability conditions of ponds. We observed changes in the water level and temporary dry ups in both agricultural and forest ponds, but differences in the time and frequency of desiccation between pond types might contribute to observed differences in the body size of adults. The agriculture in our study area is dominated by vineyards, a permanent cropland where pesticide mixtures are applied several times per year (Rossberg & Ipach 2015). Thus, soils in vineyards can be expected to be contaminated with pesticides, like it is the case for most agricultural soils in Europe (Hvězdová et al. 2018, Silva et al. 2019). Pesticides can also be detected in the agricultural ponds in our study area (Bundschuh et al. 2016, Adams et al. 2021). As negative effects of pesticides can be expected to be stronger on larvae during their development and Cusaac et al. (2017) showed a higher impact on juveniles than on adults, differences in body size might be caused by impairments during the aquatic development or early growth.

Even carry-over effects between generations can play a role. In the same area as the present study, Adams et al. (2021) sampled eggs of the common toad (*Bufo bufo*) directly after deposition from ponds with different pesticide contamination levels. The larvae were then raised in a pollution-free environment but there was still a negative correlation between the pesticide contamination level of the ponds and the size of larvae. It can thus be assumed that negative impacts are transferred from adults to juveniles. Effects on the size of larvae can also be trade-offs, e.g. from larger eggs to smaller eggs with thicker jelly coats as protection against environmental pollutions (Adams et al. 2021) or from larger eggs to smaller but more eggs.

Alternatively to carry-over effects, a selection for smaller newts in agricultural landscapes could explain differences in adult body size between pond types. Several studies showed that dispersal can select rapidly for distinct morphotypes. For example, Philips et al. (2006) showed that dispersal of cane toads (*Rhinella marina*) selects for individuals with longer legs in Australia. In Trochet et al. (2016) dispersal constraints due to landscape fragmentation resulted in Palmate Newts with shorter hindlimbs, which was explained by a higher mobility and thus higher mortality on roads of newts with longer legs. Also, in our highly fragmented agricultural landscape dispersal constraints can be expected, which makes dispersal related selection for smaller individuals possible.

Genetic structure

A larger body size of forest newts might lead to a higher dispersal ability (Phillips et al. 2006, Trochet et al. 2016, 2019). This is especially true, as our results suggest that differences in body size might already exist in juveniles, a life stage that is responsible for population connectivity in many amphibian species (Cushman 2006). Also other factors like assumed larger populations in more favourable ponds (Unglaub et al. 2018) or fewer stressors (e.g. pesticides), which could lead to a negative selection of certain haplotypes, might have an effect on the population connectivity and structure. Moreover, agriculture can act as a barrier for an amphibian species' dispersal (Lenhardt et al. 2017, Costanzi et al. 2018). Studies on the common toad also showed that amphibians tend to avoid vineyards as habitat (Leeb et al. 2020a) and that there is an avoidance behaviour against some pesticides frequently used in viticulture (Leeb et al. 2020b). Consequently, we assumed agricultural pond populations to exhibit a lower genetic diversity as well as a higher degree of inbreeding. While the higher allelic richness in forest pond populations fits our expectations, the difference in the degree of inbreeding is not strongly pronounced. In general, inbreeding is low in both pond types and the 95% confidence interval of the F_{IS} contains zero for most populations. Inbreeding is also low compared to *L. helveticus* populations in a restored pond network in northwestern France (mean F_{IS} = 0.251; Isselin-Nondedeu et al. 2017). In a French population at the Larzac Plateau a F_{IS} of -0.308 was observed, whereby this heterozygosity excess was explained by fast recolonization after a population decline (Oromi et al. 2016). In general, a high degree of inbreeding can lead to a reduced fitness of a population (Allentoft & O'Brien 2010). Thus, the absence of clear signs of inbreeding in all ponds can be rated positively. However, the relatively low number of five analyzed microsatellites might limit the detection of inbreeding. Further, we only chose populations large enough to allow the sampling of several individuals in a short time for the present study. Thus, negative effects that might occur in small agricultural populations might be overlooked. In the study area, there are several agricultural ponds that are not used by the Palmate Newt as breeding habitat, which could be a long-term result of unfavourable conditions or inbreeding depression.

Conclusions

In the present study, we showed that Palmate Newts reproducing in forest ponds are larger than newts reproducing in an intensive agricultural area. However, agriculture newts are still within the size range of newts from other European populations in more natural habitats. We could show that differences in body size most likely already existed in larvae and/or juvenile life stages and thus might be carry-over effects. This is worrying as juveniles play an important role in the population dynamics in amphibians. As we could show that forest ponds differ in several aspects from agricultural ponds, it is unclear which factor (e.g. contaminations, terrestrial habitat, microclimate, altitude, pond-specific factors like water level) is responsible for the observed effect. We found no difference in traits describing the fitness of an individual between forest and agricultural ponds, what might be the result of pond-specific differences during the breeding period caused by microclimatic conditions. The absence of a clear sign of inbreeding suggests some degree of gene flow among the agricultural pond populations. Although the Palmate Newt is considered a forest species, we conclude that agricultural ponds can be suitable breeding habitats for Palmate Newt populations. Thus, conservation efforts should aim at preserving existing agricultural ponds, but also at creating new ones. Together with green corridors between ponds, this would facilitate the dispersal of amphibians in a highly degraded agricultural landscape and reduce potential dispersal-related effects on biometric traits. Further, the potential carry-over effect indicates the need to increase the quality of the aquatic and terrestrial habitat for early life stages in agricultural landscapes, for example by ensuring a stable water level during the period of the aquatic development or reducing pollutions in and around agricultural ponds.

Additional Information and Declarations

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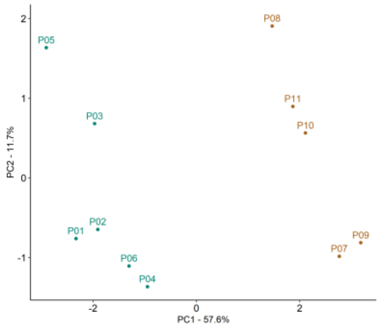
Supplementary documents



Supplementary document 1: Plot of the first and second principal component (PC). Ponds that had been classified as forest ponds are shown in teal (PC1 < 0), agricultural ponds in brown (PC1 > 0).

Supplementary document 2: Contribution of variables to principal components (PC), Eigenvalues and proportion of explained variance for each PC.

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Forest	-0.426	-0.158	-0.003	0.062	0.116	-0.196	-0.048	0.407	0.755
Altitude	-0.337	0.182	-0.529	0.123	-0.177	0.149	0.701	-0.120	0.011
Meadow	-0.169	-0.665	0.325	0.169	-0.448	0.368	0.076	-0.225	0.043
Submersed vegetation	0.192	-0.541	-0.326	-0.601	0.398	0.129	0.166	0.011	0.020
Settlement	0.330	-0.126	-0.420	0.563	0.244	0.112	-0.259	-0.392	0.299
pH	0.339	-0.089	0.455	0.327	0.376	-0.170	0.619	0.096	0.033
Conductivity	0.348	-0.250	-0.244	-0.004	-0.524	-0.687	0.074	0.068	0.013
Total agriculture	0.357	0.345	0.201	-0.387	-0.256	0.174	0.134	-0.336	0.581
Streets	0.409	0.049	-0.157	0.135	-0.235	0.493	-0.003	0.699	0.017
Eigenvalue	2.277	1.167	0.988	0.783	0.707	0.438	0.363	0.199	0.013
% exp. Var.	0.576	0.151	0.108	0.068	0.055	0.021	0.015	0.004	0.000

Supplementary document 3: Eigenvectors for each pond and principal component.

Pond-ID	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
P01	-2.332	-0.762	-1.101	-0.738	-0.269	0.772	-0.124	-0.159	-0.002
P02	-1.910	-0.647	1.502	1.032	-0.684	-0.173	-0.223	-0.293	-0.007
P03	-1.974	0.680	-0.277	-0.394	0.648	-0.616	-0.745	0.140	0.000
P04	-0.950	-1.367	0.697	-0.451	1.208	-0.085	0.483	0.086	-0.017
P05	-2.910	1.633	-1.308	0.417	-0.288	-0.297	0.554	-0.032	0.001
P06	-1.305	-1.106	0.604	0.262	-0.311	0.222	0.066	0.322	0.027
P07	2.764	-0.985	-0.732	-0.667	-1.255	-0.348	-0.056	0.132	-0.013
P08	1.464	1.907	1.109	0.238	-0.340	0.338	0.111	0.217	-0.011
P09	3.177	-0.814	-0.882	1.053	0.487	-0.332	0.129	-0.115	0.010
P10	2.110	0.563	-0.523	0.631	0.672	0.640	-0.326	-0.023	-0.004
P11	1.865	0.897	0.913	-1.384	0.133	-0.121	0.130	-0.275	0.017

Supplementary document 4: Length of the caudal filament (CF) and max. height of the tail (T) of male *Lissotriton helveticus* captured in March/April and May 2018 and the results of parametric and nonparametric tests. P-values < 0.05 are presented in bold. ^{1}As only two males were captured in May, no statistical test was performed.

Variable	Pond	March/April		May		Statistical test to compare March and May			
		mean (mm)	SD	mean (mm)	SD	Test	Test statistic (t or W)	df	p
CF	P03	4.7	1.5	5.3	2.1	t-test	-0.813	12.516	0.432
	P05	2.8	0.9	5.8	1.2	t-test	-10.862	40.804	**< 0.001**
	P07 ^{1}	5.2	1.0	1.5	0.4	-	-	-	-
T	P03	9.7	0.9	8.8	1.1	U-test	364.500	-	**0.016**
	P05	8.4	1.0	10.3	0.6	U-test	58.500	-	**< 0.001**
	P07 ^{1}	8.2	0.7	4.3	0.0	-	-	-	-

Supplementary document 5: List of all full and candidate models that were used to calculate model-averaged coefficients describing the response variables (SVL = snout-vent length; SMI = scaled mass index; T = max. height of Tail; CF = caudal filament) with the model type (GLMM = generalized linear mixed model with an Gaussian error distribution and a log-link function; LMM = linear mixed model), degrees of freedom (df), the Akaike Information Criterion with a correction for small sample sizes (AICc), the difference to the best model (ΔAICc) and the weight of a candidate model (AICc weight). Only models with a ΔAIC < 4 were considered. : = interaction between fixed effects; (1|Pond-ID) = random effect.

Model type	Response	Model	df	AICc	ΔAICc	AICc weight
GLMM	**SVL**	**~ Sex + Pond type + Submersed vegetation + Meadow + (1|Pond-ID)**	Full model			
		~ Sex + Pond type + Submersed vegetation + Meadow + (1|Pond-ID)	7	4076,4	0,00	0,603
		~ Sex + Pond type + Meadow + (1|Pond-ID)	6	4078,6	2,19	0,201
		~ Sex + Pond type + Submersed vegetation + (1|Pond-ID)	6	4079,2	2,75	0,152
LMM	**SVL**	**~ Sex + Age + Pond type + Submersed vegetation + Meadow + (1|Pond-ID)**	Full model			
		~ Sex + Age + Pond type + (1|Pond-ID)	6	650,7	0,00	0,373
		~ Sex + Age + Pond type + Meadow + (1|Pond-ID)	7	650,7	0,05	0,364
		~ Sex + Age + Pond type + Submersed vegetation + (1|Pond-ID)	7	654,4	3,70	0,059
GLMM	**Body mass**	**~ Sex + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID)**	Full model			
		~ Sex + Pond type + SVL + Pond type : SVL + Submersed vegetation + (1|Pond-ID)	8	-241,6	0,00	0,307
		~ Sex + SVL + Submersed vegetation + (1|Pond-ID)	6	-240,1	1,43	0,150
		~ Sex + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID)	9	-240,0	1,61	0,137
		~ Sex + Pond type + SVL + Submersed vegetation + (1|Pond-ID)	7	-239,6	1,95	0,116
		~ Sex + Pond type + SVL + Pond type : SVL + (1|Pond-ID)	7	-238,7	2,89	0,072
		~ Sex + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	7	-238,2	3,36	0,057
		~ Sex + Pond type + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	8	-237,9	3,67	0,049
GLMM	**Body mass**	**~ Sex + Age + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID)**	Full model			
		~ Sex + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	7	-7,2	0,00	0,186
		~ Sex + Age + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	8	-6,8	0,42	0,151
		~ Sex + SVL + Submersed vegetation + (1|Pond-ID)	6	-6,3	0,96	0,116
		~ Sex + Age + SVL + Submersed vegetation + (1|Pond-ID)	7	-5,6	1,65	0,081
		~ Sex + Pond type + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	8	-5,0	2,18	0,063
		~ Sex + Age + Pond type + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	9	-4,6	2,67	0,049
		~ Sex + Pond type + SVL + Submersed vegetation + (1|Pond-ID)	7	-4,1	3,17	0,038
		~ Sex + SVL + Meadow + (1|Pond-ID)	6	-3,7	3,56	0,031
		~ Sex + Pond type + SVL + Meadow + (1|Pond-ID)	7	-3,3	3,91	0,026
		~ Sex + Age + Pond type + SVL + Submersed vegetation + (1|Pond-ID)	8	-3,3	3,91	0,026
		~ Sex + Pond type + SVL + (1|Pond-ID)	6	-3,3	3,95	0,026
GLMM	**SMI**	**~ Sex + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID)**	Full model			
		~ Sex + SVL + Submersed vegetation + (1|Pond-ID)	6	-296,8	0,00	0,239

		~ Sex + Pond type + SVL + Submersed vegetation + (1|Pond-ID)	7	-296,6	0,22	0,215
		~ Sex + Pond type + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	8	-295,7	1,15	0,134
		~ Sex + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	7	-294,8	2,03	0,087
		~ Sex + Pond type + SVL + Pond type : SVL + Submersed vegetation + (1|Pond-ID)	8	-294,6	2,22	0,079
		~ Sex + SVL + (1|Pond-ID)	5	-294,0	2,82	0,058
		~ Sex + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID)	9	-293,7	3,18	0,049
		~ Sex + Pond type + SVL + (1|Pond-ID)	6	-293,6	3,22	0,048
		~ Sex + Pond type + SVL + Meadow + (1|Pond-ID)	7	-293,1	3,70	0,038
GLMM	**SMI**	**~ Sex + Age + Pond type + SVL + Submersed vegetation + Meadow + (1|Pond-ID)**		Full model		
		~ Sex + Age + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	8	-20	0,00	0,330
		~ Sex + Age + Pond type + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	9	-18,2	1,75	0,137
		~ Sex + Age + SVL + Submersed vegetation + (1|Pond-ID)	7	-17,3	2,65	0,087
		~ Sex + Age + Pond type + SVL + Pond type : SVL + Submersed vegetation + Meadow + (1|Pond-ID)	10	-16,7	3,24	0,065
		~ Sex + Age + SVL + Meadow + (1|Pond-ID)	7	-16,5	3,49	0,058
		~ Sex + SVL + Submersed vegetation + Meadow + (1|Pond-ID)	7	-16,0	3,95	0,046
LMM	**T**	**~ Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID)**		Full model		
		~ SVL + SMI + (1|Pond-ID)	5	1196,7	0,00	0,617
		~ Pond type + SVL + SMI + (1|Pond-ID)	6	1198,2	1,50	0,291
LMM	**T**	**~ Age + Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID)**		Full model		
		~ Pond type + SVL + SMI + (1|Pond-ID)	6	210,6	0,00	0,267
		~ SVL + SMI + (1|Pond-ID)	5	210,8	0,21	0,240
		~ Pond type + SVL + SMI + Meadow + (1|Pond-ID)	7	211,4	0,81	0,178
		~ SVL + SMI + Meadow + (1|Pond-ID)	6	211,9	1,32	0,138
LMM	**CF**	**~ Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID)**		Full model		
		~ Pond type + SVL + SMI + (1|Pond-ID)	6	1440,3	0,00	0,386
		~ SVL + SMI + (1|Pond-ID)	5	1441,1	0,75	0,265
		~ SVL + SMI + Submersed vegetation + (1|Pond-ID)	6	1442,8	2,50	0,111
		~ Pond type + SVL + SMI + Submersed vegetation + (1|Pond-ID)	7	1443,5	3,14	0,080
		~ Pond type + SVL + Pond type : SVL + SMI + (1|Pond-ID)	7	1444,3	3,99	0,053
LMM	**CF**	**~ Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID)**		Full model		
		~ Pond type + SVL + SMI + Meadow + (1|Pond-ID)	7	240,6	0,00	0,143
		~ Pond type + SVL + SMI + (1|Pond-ID)	6	240,6	0,01	0,143
		~ Pond type + Meadow + (1|Pond-ID)	5	242,3	1,68	0,062
		~ SVL + SMI + (1|Pond-ID)	5	242,6	1,98	0,053
		~ Pond type + (1|Pond-ID)	4	242,6	2,00	0,053
		~ (1|Pond-ID)	3	242,8	2,21	0,047
		~ SVL + SMI + Meadow + (1|Pond-ID)	6	242,9	2,23	0,047
		~ Pond type + SVL + Meadow + (1|Pond-ID)	6	243,1	2,50	0,041
		~ Pond type + SMI + Meadow + (1|Pond-ID)	6	243,4	2,82	0,035
		~ Pond type + SVL + (1|Pond-ID)	5	243,5	2,83	0,035
		~ Meadow + (1|Pond-ID)	4	243,6	2,99	0,032
		~ Pond type + SMI + (1|Pond-ID)	5	243,7	3,06	0,031
		~ SMI + (1|Pond-ID)	4	243,8	3,21	0,029
		~ SVL + SMI + Submersed vegetation + Meadow + (1|Pond-ID)	7	244,5	3,90	0,020

Supplementary document 6: Results from testing for linkage disequilibrium for each locus pair across all populations with GENEPOP 4.6 (Fisher's method). No linkage disequilibrium was found (all p-values > 0.05).

Locus pair			chi^2	Df	p
Lh7	&	Lh44	11.759	20	0.924
Lh7	&	Us9	14.909	22	0.866
Lh44	&	Us9	13.841	20	0.838
Lh7	&	Lh16	18.250	22	0.691
Lh44	&	Lh16	19.766	20	0.473
Us9	&	Lh16	15.899	22	0.821
Lh7	&	Lh19	4.498	8	0.810
Lh44	&	Lh19	6.830	6	0.337
Us9	&	Lh19	6.210	8	0.624
Lh16	&	Lh19	4.303	8	0.829

Supplementary document 7: Results from testing each population and Loci for Hardy-Weinberg equilibrium (HWE). NA = test was not possible because there is only one allele in the population. P-values < 0.05 are presented in bold.

Loci	**P01**	**P02**	**P03**	**P04**	**P05**	**P06**	**P07**	**P08**	**P09**	**P10**	**P11**
Lh7	0.918	0.455	0.723	0.729	0.922	0.202	0.289	0.065	0.488	0.910	0.957
Lh44	0.993	0.892	0.633	0.880	0.741	0.789	NA	0.477	0.952	0.807	0.507
Us9	0.314	0.295	0.324	0.636	0.429	0.512	0.709	0.098	0.180	0.441	0.311
Lh16	0.404	0.974	0.919	**0.001**	0.440	0.775	0.899	0.809	0.411	0.411	0.238
Lh19	NA	0.699	0.740	NA	NA	NA	0.807	NA	0.920	NA	NA

3. Summary of the results

In chapter 1 we showed that common toads can be found in vineyards during their post-breeding migration, although they tend to avoid them, and that they have a high overall risk of being exposed to pesticides. Initially, we expected most toads to migrate to the nearby Palatinate Forest after breeding, because this forest is commonly considered as the most suitable terrestrial habitat for common toads in the study area. However, a large part of the population stayed in the agricultural landscape over large parts of the year, and only 4 out of 51 individuals reached the forest. Toads spent about 15% of the time directly in vineyards. However, in general, toads tend to avoid vineyards as habitat, as movements ended less often there than expected from a random habitat choice. By combining telemetry data with information about pesticide applications from local wine growers and taking spray drift and the half-life values of applied pesticides into account, we estimated that on a single day up to 24% of the whole breeding population came into contact with pesticides, resulting in a high overall exposure risk.

In the experiment described in chapter 2, we found evidence for the avoidance of juvenile common toads against some frequently used viticultural pesticides at field rates. This was achieved by performing arena choice experiments in the laboratory, whereby five pesticides, one herbicide and one insecticide with 10% or 100% of the maximum recommended field rate (FR_{max}) were tested with 16 replicates per test run. In six out of seven tested pesticides, the toads spent more time on the uncontaminated side of the arena at FR_{max}. However, this avoidance of the contaminated side was only significant for the folpet formulation Folpan® 500 SC, the metrafenone formulation Vivando® and the glyphosate formulation Taifun® forte at FR_{max}. For Wettable Sulphur Stulln (sulphur) a trend to avoidance was detected. No avoidance was observed when testing Folpan® 80 WDG (folpet), Funguran® progress (copper hydroxide) and SpinTorTM (spinosad). Moreover, for 10% of FR_{max} of all the tested formulations no avoidance was detected. In the choice tests in which we observed avoidance behaviour, the median distance a toad moved on the uncontaminated side was significantly larger than on the contaminated side. Subsampling the data set to one sample every 15 or 60 minutes, which corresponds to a reduction of the sampling frequency during the data acquisition, led to a similar avoidance pattern. Although lowering the sampling frequency comes with the risk of overlooking weak avoidance behaviour, it might be a solution when tracking software can not be used or is not available. Analysing only the data from the first hour of an experimental run instead of the full 24 hours did not allow for the detection of any avoidance behaviour.

In chapter 3 we showed that gene flow between *Rana temporaria* populations located within viticultural areas is lower than between populations located in the Palatinate Forest, suggesting that vineyards have to be considered as a barrier for amphibians. Median pairwise F_{ST} values were between 0.0215 at a distance of 2.34 km between populations and 0.0987 at 2.39 km for agricultural populations and 0.0041 at 5.39 km to 0.0159 at 9.40 km for populations located in the Palatinate Forest. Landscape genetic analyses suggest that the observed genetic differentiation between populations is not a result of isolation by geographic distance. However, weighted distance models provide evidence that vineyards limit the gene flow between *R. temporaria* populations and thus contribute to landscape fragmentation, even more than settlements or streets with associated traffic intensity. Population structure analysis confirmed reduced gene flow within the agricultural landscape as three agricultural populations were identified as separate clusters, suggesting their isolation from the meta-population.

In chapter 4 we could demonstrate that the pesticide contamination level in a breeding pond has a significant effect on the reproductive capacity of common toads. In total 22 different pesticides were detected with 0 to 19 pesticides per pond. The pesticide contamination per pond (sum of toxic units) was highly correlated with the proportion of agricultural land use around the pond. Along this pesticide gradient effects on the reproductive capacity were found. From eggs from more contaminated ponds fewer tadpoles hatched (decreased fertilization rate) and their survival rate as well as size at Gosner Stage 25 was decreased. However, toads reproducing in more contaminated ponds laid more eggs (i.e. showed an increased fecundity), resulting in a comparable overall fitness between populations.

In the field study of chapter 5, palmate newts captured in agricultural ponds had a smaller body size than those from forest ponds, which might be related to a suboptimal habitat quality for early life stages. As amphibians grow throughout their lifetime, differences in the demographic structure with older individuals in forest ponds would explain the differences in body size. However, skeletochronology data revealed no difference in the median age or growth between habitat types but suggest that differences in the size of adults might be carry-over effects from subadult life stages or a trade-off. Body mass, body condition and sexual dimorphic traits (length of the caudal filament and max. height of the tail) correlated with body size, but no additional effect of the habitat type was found. Genetic analyses showed a higher allelic richness in forest pond populations, but no clear sign of inbreeding in any agricultural population. Thus, there might be at least some degree of gene flow between populations in the agricultural landscape. Individuals from agricultural ponds were within the size range of newts from other European populations in more natural habitats.

4. Discussion

Today, agriculture is the most dominant landscape type in many parts of the world. It is linked to intensive pesticide use, habitat loss and fragmentation, which are named as some of the main drivers in the worldwide amphibian decline. Thus, understanding the situation of amphibians in the agricultural landscape is crucial for conservation measures. In this thesis, I found evidence for amphibian avoidance of pesticide contaminations, indications of (chemical) landscape fragmentation as well as adverse effects of amphibian development in the agriculture landscape.

4.1. Agriculture as habitat for amphibians

It is well known that water bodies in agricultural landscapes can function as reproduction habitats for amphibians (Beja and Alcazar, 2003; Berger et al., 2013; Knutson et al., 2004; Lenhardt et al., 2013). The importance of remaining agricultural ponds has been emphasized by reporting of a general pond loss during the last decades (Curado et al., 2011). In the investigated vineyard-dominated study area in Southern Palatinate, drainage and rain retention ponds are used by amphibians for breeding. In chapters 1-5, a total of 13 amphibian breeding ponds within or near agriculture were studied, with additional 9 ponds in the forest. In the same area, Bischoff (2008) mapped 75 ponds and documented amphibian populations in 88% of all water bodies with an average of 1.8 species per pond. During several field trips and mappings as study preparation for this thesis, dozens of additional amphibian populations could be documented. Apparently, there are many amphibian populations present in the agricultural landscape in Southern Palatinate. Although a permanent crop like wine with greened rows and shade during the summer might be more suitable for amphibians than other types of arable land, this does not necessarily mean that they are ideal habitats. Bischoff (2008) found 11 amphibian species in the area, but some species with higher habitat requirements, such as the European tree frog (*Hyla arborea*) or the common midwife toad (*Alytes obstetricans*) were not detected, despite being generally documented in this part of Germany. Furthermore, the northern crested newt (*Triturus cristatus*) was only found in 2 out of 75 ponds, and both ponds were not directly located in vineyards. Agricultural landscapes and all their linked processes might thus shape the occurrence and distribution of amphibian species. Although the effect of agriculture on occurence is often said to be negative (Beja and Alcazar, 2003; Boissinot et al., 2019; Piha et al., 2007; Ray et al., 2002; Suárez et al., 2016), it might depend on the species in focus (Koumaris and Fahrig, 2016; Trochet et al., 2016a).

The presence of amphibians in an agricultural pond does not necessarily imply that the population is stable and healthy. For example, in chapter 3 the number of common frog breeding pairs in agricultural ponds was too small to sample enough adults, so that a novel methodological approach had to be developed to analyse the microsatellite data from siblings of the same clutch for reliable population structure and gene flow estimations (Lenhardt and Theissinger, 2017). In contrast, common frog populations in the near Palatinate Forest can consist of thousands of breeding pairs. Agriculture can further affect morphological traits of amphibians. For example, Guillot et al. (2016) showed that common toads from intensive agriculture were larger than toads from forest habitats, but less symmetrical, which indicates environmental stress during early life stages. In the study on palmate newts (chapter 5) we found smaller adults in agricultural ponds, which might be linked to suboptimal conditions for larvae and/or juveniles in or around the ponds.

Reasons for negative effects of agriculture are manifold and, in most cases, exact cause-effect relationships cannot be identified, especially in field studies. Reduced population sizes in agricultural amphibian populations might be caused by direct mortality of individuals due to agricultural processes like mechanical tillage (Pfeffer et al., 2011) or mowing (Humbert et al., 2009). In fact, three common toads that were tracked via telemetry (chapter 1) died because of such agricultural processes, two of them directly in vineyards. Moreover, pesticides that are frequently applied in agricultural areas can have lethal effects. When amphibians are directly oversprayed with pesticides high mortality rates, in some cases of up to 100%, can be observed at field application rates (Belden et al., 2010; Brühl et al., 2013; Relyea, 2005). A recent laboratory study showed, that even contact with contaminated soil can result in the death of juvenile common frogs (Adams et al., 2021a). Besides acute effects, chronic and sublethal effects can affect populations, for example due to impairment of the reproduction capacity as shown in chapter 4.

With our telemetry study (chapter 1) we could show that common toads spent some time directly in vineyards and are therefore exposed to the dangers linked to agriculture mentioned above. However, they generally tend to avoid vineyards as habitat and preferred remnant structures like bushes, groves and fallows. Thus, we conclude that vineyards are in general suboptimal habitats for toads and therefore less often used, especially in spring when the absence of shading vine leaves results in an unfavourable microclimate for toads. Also several other studies documented an avoidance of agricultural fields. For example, Vos et al. (2007) showed that arable land was the most avoided type of habitat when studying the effect of landscape composition on the small-scale migration of juvenile common frogs around a pond in the Netherlands. Crops were also the least preferred habitat in a telemetry study on the terrestrial habitat use of the natterjack toad (*Epidalea calamita*) in Spain (Miaud and Sanuy, 2005). Salazar et al. (2016) detected 91 common toads around a pond in an agricultural landscape in the UK, but not a single individual was found directly in a cultivated field. Although overall still little is known about the terrestrial fine-scale habitat use of European amphibians in agricultural landscapes, these findings are in line with our results and highlight the importance of remnant structures in agricultural landscapes and green corridors between populations.

4.2. Pesticide exposure

Pesticides are applied on agricultural fields to protect crops from different kinds of pests like insects or weeds. In our vineyard-dominated study area mainly fungicides are used against pathogenic fungi and oomycetes, whereby up to 12 pesticide applications per year were reported by local farmers (chapter 1). In chapter 4 we detected up to 19 different pesticides in a single pond, confirming that spray drift and run-off lead to the contamination of water bodies within or near agricultural fields. As a consequence, amphibians with their complex, biphasic life cycle can be exposed to pesticides during all life stages. While it is comparatively easy to analyse the actual exposure of aquatic life stages (i.e. both pesticides and eggs/larvae/adults can be detected in the pond at the same time), this is much more complex for terrestrial life stage. Some studies showed that amphibians can be found directly in arable fields (Knutson et al., 2004; Müller et al., 2013; Schweizer, 2014), but only a few combined fine-scale data on habitat use with data on pesticide applications (Berger et al., 2013; Lenhardt et al., 2015). Thus, there is only little information about the spatial-temporal overlap of pesticide applications with the terrestrial activity phase of temperate amphibians. This is concerning, because such data are critical to estimate the actual risk to amphibians posed by pesticide exposure.

This highlights the importance of our study in chapter 1, where for the first time telemetry data were combined with pesticide application data. We showed that toads spent some time directly in vineyards and can be present during a pesticide application. In general, common toads are primarily nocturnal, usually hide during the day and are then often covered with soil or vegetation. Their risk of being directly oversprayed without interception might therefore be generally low. However, we found individuals during the day outside their hiding place, just before a pesticide application, and we observed multiple application events after sunset. Consequently, direct overspray might be a rare worst-case scenario, but still realistic for adult common toads in our study area. During fieldwork we also observed hundreds of metamorphs directly in vineyards. Unlike adults, metamorphs are diurnal and their risk of being directly oversprayed is comparably higher. This is alarming, as juveniles are generally more sensitive to pesticides than adults (Cusaac et al., 2017). As pesticides do not break down immediately and pesticides are applied several times each year (chapter 1, Roßberg, 2013), most agricultural soils are contaminated over large parts of the year with several pesticides (Hvězdová et al., 2018; Silva et al., 2019). Taking the persistence of a pesticide in the environment (half-time of a pesticide, DT_{50}) and spray drift into account, it becomes clear that a large part of the common toads came in contact with contaminated soil during their terrestrial life stage.

While hiding in the soil might reduce the risk of being directly oversprayed, contact with contaminated agricultural soil with the whole body might even increase a potential uptake. The negative effects of amphibian skin contact with contaminated soil were shown in Adams et al. (2021a). Up to 60% of juvenile *R. temporaria* that were placed on soil contaminated with a folpet formulation died, and surviving individuals suffered from sublethal effects on the locomotor activity as well as on feeding behaviour. In chapter 2 we exposed juvenile common toads to soils that were oversprayed with different pesticides frequently used in German vineyards. As no mortality in the test species was observed, lethal effects might be species and/or substrate dependent. However, we found a behavioural response to the exposure, as the juvenile common toads avoided the contamination in an arena choice experiment. This avoidance of contaminated areas might lead to a decreased exposure of individuals. However, it might also result in adverse effects on the meta-population level as agricultural areas with contaminated soils could act as a barrier during migration/dispersal. Yet, it remains unclear whether the observed avoidance can be transferred from the laboratory to the field. Also the impairments of the reproduction capacity of common toads in chapter 4 suggest long-term pesticide exposure in their aquatic and/or terrestrial habitat. Adverse environmental conditions for larval/subadult palmate newts that might result in carry-over effects to later stages (chapter 5) might be linked to pesticide exposure in the aquatic or terrestrial habitat too. In summary, our results indicate that the overall risk for amphibians to be exposed to pesticides must be considered as very high in agricultural landscapes, necessitating a reconsideration of intensive pesticide use.

4.3. (Chemical) landscape fragmentation and its challenges

A landscape consists of a mosaic of elements, whereby some are used by a species in focus and some are not. When certain elements are avoided or cannot be crossed (e.g. highways), suitable habitats might be isolated from each other, a process that is referred to as landscape fragmentation. An indirect way to study landscape fragmentation is to link the gene flow between populations to (traditionally mainly visible) spatial features describing the landscape (i.e. landscape genetics). For example, landscape genetics were used to identify arable land as a barrier and the Loire River in Western France as a corridor for the marbled newt (*T. marmoratus*) (Costanzi et al., 2018). Another example is a study of Van Buskirk (2012), in which landscape genetics were used to show that highways cause a 40% reduction of gene flow between alpine newt (*Ichthyosaura alpestris*) populations in the Switzerland. However, there are some limitations and pitfalls when using landscape genetic methods. Van Strien (2017) discussed the important role of the topology (i.e. the spatial arrangement of sampled populations) in landscape genetic studies and pointed out that ideally all populations in a study area should be sampled, that dispersal limitations of the focal species should be considered in the analysis and that it may be advantageous to consider only selected and not all possible population pairs in linked-based analysis.

The mentioned limitations are also the main reasons why we did not use linked-based landscape genetics to study the gene flow between *L. helveticus* populations in chapter 5. Here, the geographic distances between the sampled populations were 910 to 23,770 m (mean = 10,300 m), while typical migration distances of the palmate newt are only several hundred meters (Jehle and Sinsch, 2007; Lenhardt et al., 2013). Instead, we calculated the allelic richness and the inbreeding coefficient for each population and compared these population genetic parameters between forest and agricultural populations. We found that populations located in forests had a higher allelic richness, which could indicate a higher gene flow between forest populations compared to agricultural populations. When agriculture leads to a strong isolation of agricultural ponds and a strong reduction in gene flow, this should be also reflected in the degree of inbreeding of isolated populations. Also the landscape model of Lenhardt et al. (2013) suggested strong isolation of some of the agricultural newt populations. Surprisingly, we did not find a clear sign of inbreeding in any agricultural population, suggesting at least some gene flow between them. Several factors could contribute to the higher than expected gene flow. On the one hand, one might just underestimate the migration capacity of newts and amphibians in general. Migrations over 34 km were reported for toads (Smith and Green, 2006), and it is known that the invasion front of the cane toad (*Rhinella marina*) in Australia expands up to 55 km per year (Phillips et al., 2007). Sinsch (2012) used telemetry data and migration capacity models to show that breeding ponds of the natterjack toads in distances up to 12 km are still connected, while observation data suggest that typical migrations are below 5 km for this species (Jehle and Sinsch, 2007). In general, only little is known about the dispersal abilities of amphibians. This is especially true for newt species, for which relevant studies on migration and dispersal behaviour are often completely lacking. Unfortunately, there is even a lack of methods to study these life history aspects. For example, telemetry, which can be used to study the migration of anurans (chapter 1), is hardly used for Urodela (but see e.g. Schabetsberger et al. (2004) or Dervo et al. (2010)) and never used for small newts like from the genus *Lissotriton*. On the other hand, one might also underestimate the number of populations present in an area and thus overestimate the degree of isolation of certain populations. This is especially true near settlements, where garden ponds on private property can be important habitats for amphibians (Glandt, 2018), but are usually inaccessible and cannot be considered in field investigations. When studying amphibians near settlements, there is also the problem that many garden owners relocate amphibians from their gardens to publicly accessible ponds (e.g. because they are disgusted by them; Banks and Laverick, 1986; Pavol and Fančovičová, 2012; Prokop et al., 2016) or from known amphibian ponds to their gardens (e.g. to enrich the local biodiversity; Banks and Laverick, 1986). In both cases, amphibians are often transferred over distances that exceed the natural migration capacity of a species or are helped to overcome barriers that usually limit migration. Such translocations, which are also common in our study area, might affect the results of landscape genetic studies.

Methods used in chapter 3 and chapter 5, but also in e.g. Johansson (2004), Frei (2016) or Constanzi (2018), aim to explain population genetic parameters with landscape characteristics and thus to make inferences about the potential of agricultural landscapes to act as barriers and lead to landscape fragmentation. However, they do not allow for making statements about a chemical landscape fragmentation, i.e. whether agricultural chemicals (i.e. pesticides) contribute to the fragmentation of the landscape. In theory, both land use and pesticide use can be included in landscape genetic models, but there are two major pitfalls. First, data about land use but also pesticide applications, for the whole or at least most parts of a study area, are needed. Second, agricultural areas with a mosaic of different management types, ranging from no pest control to very intensive pesticide use, are needed. While data about land use that allows for determining the proportion of agricultural areas between populations can be received via remote sensing and are often available (e.g. ATKIS DLM50, which was also used in chapters 3-5), data about pesticide applications are usually lacking. The viticulture landscape in our study area is managed by hundreds of farmers. Although they have to keep track of their pesticide applications, they do not have to provide them for scientific studies. For the telemetry study (chapter 1) we tried to get information about pesticide applications from local farmers for all vineyards in the study area. Although dozens of farmers were contacted, we failed to get the actual application data for even most vineyards and partly had to resort to data from farmers in the surroundings of our study site. This still allowed for the estimation of the overall exposure risk of the population, but not the actual exposure of every individual for every day. Moreover, this data were also not sufficient for modelling in a landscape genetic study. Consequently, future landscape genetic studies that aim to investigate chemical landscape fragmentation should take place in well-chosen regions where all stakeholders are known and agree to provide the pesticide application data. In viticulture, where agricultural fields are often small-scaled, the owner structures and management regimes can be quite complex and diverse. For example, three rows of vine from farmer A with intensive pesticide use can be next to three rows of vine from farmer B that are organically managed. Such a scenario does not fit a simple model crop type to study chemical landscape fragmentation. Other crops in areas like Eggersdorf (north-east of Germany), where the exposure to pesticides of four temperate amphibian species during their pre- and post-breeding migration was studied based on application data of only seven farmers that are managing an area of 2,850 ha (Berger et al., 2013), might represent more suitable setups.

Even if chemical landscape fragmentation cannot be proven straight forwards, our studies provide some indications for this scenario. The avoidance of vineyards (chapter 1) and the reduced gene flow observed in chapter 3 indicates some degree of general landscape fragmentation in our study area. The avoidance of contaminated areas (chapter 2) can be seen as a hint that pesticides contribute to this fragmentation. In addition, pesticides might enhance the negative effects of fragmentation when pesticide exposure leads to a reduced number of migrating individuals in agricultural areas. In chapter 4, the reduced number of hatched tadpoles and decreased survival rate to Gosner stage 25 as a consequence of pesticide exposure were compensated by an increased number of eggs. However, surviving larvae were also smaller, which might result in smaller juveniles/adults and thus might influence the long-term survival and the migration capacity of a population. In conclusion, the combination of landscape fragmentation and pesticide contamination might reduce the fitness and thus long-term survival probability of populations in the studied area.

4.4. Implications for risk assessment and conservation measures

In chapter 2 and chapter 4 we showed potential negative effects of pesticides for amphibians, not only on the individual but on the population and meta-population level. Although this is in line with several previous studies that demonstrated adverse effects of pesticides (reviewed by e.g. Baker et al., 2013; Brühl et al., 2011; Sievers et al., 2019), amphibians are not yet considered in the risk assessment of pesticides in the EU. To estimate the risk of amphibians, surrogate species are used with toxicity data for fish being discussed to cover the risk for aquatic and data for birds and mammals the terrestrial life stages of amphibians (Ortiz-Santaliestra et al., 2018). However, this approach neglects the special characteristics of amphibians like the complex metamorphosis of larvae or their general ectothermic nature. While a recent study showed that surrogate species like the rainbow trout (*Oncorhynchus mykiss*) can cover the risk for aquatic life stages of Central European species when the uncertainty factor generally applied in the fish risk assessment is used (Adams et al., 2021b), there are concerns about the terrestrial life stage of amphibians (Brühl et al., 2011). The risk assessment of pesticides for mammals and birds is mainly based on the oral uptake of a substance, which might cover the oral exposure route in amphibians (Crane et al., 2016). Although this exposure pathway also plays a role in amphibians, the dermal uptake is considered to be more relevant. Amphibians have highly permeable skin (Kaufmann and Dohmen, 2016), which favours the uptake of water but also larger molecules (Llewelyn et al., 2016). In contrast, birds and mammals have feathers and fur, respectively, which protect them from dermal exposure. Thus, toxicity data from mammals and birds might not cover terrestrial amphibians completely (Ortiz-Santaliestra et al., 2018). Besides the use of surrogate species, there are also general weaknesses in the current scheme for environmental risk assessment, which will most likely also be found in a future risk assessment for amphibians. For example, indirect effects of pesticides on populations, interactions between trophic levels and realistic pesticide application scenarios (sequences of applications with mixtures of pesticide formulations) are largely ignored (Brühl and Zaller, 2019). Moreover, effects like the avoidance of contaminated areas shown in chapter 2 would most likely be overlooked by current pesticide risk assessment approaches. Until appropriate guidelines are established and the risk of already approved pesticides is re-evaluated, the logical conclusion under the precautionary principle would be to manage agricultural areas completely without pesticides. Unfortunately, this is usually not feasible in viticulture. Still, it might be worth it to refrain pesticide applications when amphibians are generally more active (e.g. during or shortly after rain events or after sunset) or when juveniles emerge out of the water. As we observed high plasticity in the post-breeding migration (chapter 1), such a temporary renunciation of pesticides does not, however, eliminate the exposure risk completely, especially as some pesticides are persistent and do not break down immediately after application.

One approach to reduce the risk of pesticides on non-target organisms like amphibians is to replace synthetic pesticides with organic substances as it is the case in organic farming. Unfortunately, also the effects of most organic plant protection products on terrestrial amphibians remain unclear, as data on toxicity are lacking. In chapter 2, we tested four conventional and three organic pesticide formulations in an arena choice experiment. Although we only found a significant avoidance of areas that were oversprayed with the three conventional formulations, there was also a trend of avoidance of sulphur, which is often used in organic farming. Thus, also vineyards with organic management might contribute to chemical landscape fragmentation and might have negative effects on the population- and meta-population level. The lack of avoidance of the copper formulation Funguran® progress might have both positive and negative effects. On the good side, this means that copper, which is often used as a replacement for conventional fungicides (Gessler et al., 2011) and can thus be found in vineyard soils (Steinmetz et al., 2017), might not contribute to landscape fragmentation. On the other hand, the lack of avoidance results in potential exposure to soils contaminated with copper. Although we could not observe direct mortality in common toads exposed to the maximum recommended field rate of Funguran® progress, sublethal effects cannot be ruled out. Azizishirazi et al. (2021) concluded in a review that amphibians are comparably tolerant against acute, but sensitive to chronic copper exposure. Although this conclusion is based on data on aquatic exposure, it might also be transferred to the terrestrial life stage. Copper is frequently applied in German vineyards, making an accumulation of copper in soils and thus a chronic exposure of amphibians in agricultural fields likely. In organic vineyards in Southern Palatinate, copper concentrations are two times higher than in conventional vineyards (Steinmetz et al., 2017), which is higher than the lethal copper concentration for terrestrial red-backed salamanders (*Plethodon cinereus*), one of the few amphibian species for which toxicity experiments with a terrestrial life stage and copper were conducted (Bazar et al., 2009). The general trend towards organic farming (Willer and Lernoud, 2019), which can also be observed in viticulture (Provost and Pedneault, 2016; Willer and Lernoud, 2008), is considered to be advantageous for biodiversity (Hole et al., 2005; Puig-Montserrat et al., 2017). However, the high number of organic fungicides can also have negative effects in some cases (Reiff et al., 2021). In general, organic farming might be also advantageous for amphibians, but there is a lack of studies to prove that (Randall and James, 2012). The risk of pesticides formulations used in organic farming should thus also be evaluated in more detail in the future.

Besides replacing conventional with organic pesticides, numerous studies proved that the general amount of applied pesticides can be reduced drastically (Pertot et al., 2017). For example, fungicides, the most frequently used pesticide class in Central European vineyards that are preventively used to protect the plants primarily from powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*) (Roßberg and Ipach, 2015), can be reduced by planting vine varieties that are resistant or at least tolerant against these diseases (Fuller et al., 2014). Furthermore, services like "VitiMeteo" (Bleyer et al., 2008) use weather data and models for leaf development of vine to forecast the occurrence of downy mildew in order to recommend wine farmers the optimal timing of fungicide applications. Another example is the smartphone app "DOSAVIÑA" (Gil et al., 2019) which calculates the optimal pesticide concentration and application volume based on the leaf density and the sprayer type, resulting in a reduction of the amount of applied pesticides by 20%. The use of herbicides can usually be completely abandoned in vineyards and replaced by cover cropping and tillage. In many regions, insecticides are frequently used, e.g. against the vine moth (*Eupoecilia ambigu*ella) or the American grapevine leafhopper (*Scaphoideus titanus*), but play only a minor role in German vineyards. In some cases, mating disruption methods are used to control insect populations (Harari et al., 2007; Louis and Schirra, 2001). Several studies showed that a complex landscape with semi-natural habitats promotes natural pest control. For example, Barbaro et al. (2017) demonstrated that the management type of vineyards, but also the habitat heterogeneity in the surrounding landscape supports the occurrence of insectivorous birds. Also for the abundance of bats, the structural heterogeneity around vineyards, i.e. the presence of habitats like shrubs or trees, is important (Charbonnier et al., 2021). Such remnant patches further increase the abundance of predatory invertebrates (Thomson and Hoffmann, 2009) and are often used by amphibians like the common toad as terrestrial habitat (chapter 1). In general, amphibians can play an important role for natural pest control in many types of agriculture (Civantos et al., 2012; Valencia-Aguilar et al., 2013), but studies on their relevance in German vineyards are lacking.

In wealthy countries with a high income, a reduction of the amount of applied pesticides can often be achieved without a decrease of productivity (i.e. crop output per hectare) (Schreinemachers and Tipraqsa, 2012). However, taking also middle- and low-income countries into account, a correlation between productivity and pesticide use can be found, with an increase of 1.8% of pesticide use being associated with an increase of 1% of crop output per hectare (Schreinemachers and Tipraqsa, 2012). Therefore, policies should be established to ensure that a decrease in productivity after a reduction of pesticides is not compensated by an increase of agricultural areas e.g. by deforestation (Grau et al., 2005; Winkler et al., 2021) or drainage of peatlands (Swindles et al., 2019).

Amphibians play a crucial role in aquatic and terrestrial food webs and represent an important link between these two habitats. As they are the most endangered species group, comprehensive conservation measures are required. In agricultural landscapes, the need for conservation measures is emphasised by the combination of landscape fragmentation due to avoidance of agricultural areas, frequent pesticide exposure in the terrestrial and aquatic habitat as well as resulting adverse effects on individual physiological development. Remnant structures like bushes, groves, fallows and of course also water bodies must be preserved and their quality as aquatic or terrestrial habitat be increased, especially for early life stages (chapter 5). In our study on the habitat use of the common toad, 95% of all relocations were within a distance of 756 m to the pond (chapter 1). This corresponds with the results of a meta-analysis of Rittenhouse and Semlitsch (2007) in which frogs had a 95% probability to be found within a distance of 703 m (664 m when including newts), and most individuals were found in a distance of 30 to 200 m. The area in a distance of up to 200 m might thus be the most relevant for conservation measures. However, Rittenhouse and Semlitsch (2007) also concluded that relevant distances to a pond are species-specific and that measures have to ensure a successful migration between different habitats when they are clumped in the landscape. Creating new habitat patches around a pond would increase landscape complexity and lead to a mosaic of habitats in favour of amphibians. Green corridors between populations and different habitats would allow migrating individuals to avoid agricultural and thus contaminated areas (chapter 1 & 2) and to reduce their exposure risk (chapter 1) while preventing the fragmentation of the landscape and thus the isolation of populations (chapter 3). Non-crop habitats around a pond, and of course a general reduction of pesticides, would further reduce the pesticide contamination level in a pond and thus sublethal effects like impairments of the reproduction capacity (chapter 4). The call for more heterogeneity and green corridors to protect amphibians in agricultural landscapes is an echo to several other amphibian studies on this topic (e.g. Boissinot et al., 2019; Ghosh and Basu, 2020; Guerra and Aráoz, 2015; Hartel et al., 2009; Salazar et al., 2016; Sawatzky et al., 2019; Vos et al., 2007).

5. Perspective & future studies

All studies presented in this thesis are case studies focusing on only a few populations of a few species in only one type of agriculture, which might limit the generalization of our results. Thus, further studies are needed which extend the methodological approaches to get a deeper understanding of the situation of amphibians in agricultural landscapes and to get basic data that can be used for developing a general pesticide risk assessment for amphibians.

To estimate the pesticide exposure risk of European amphibians in agricultural landscapes, more studies on the spatial-temporal overlap of pesticides and amphibians in the field should be conducted. Studies should take place in different types of agriculture, whereby study areas should be chosen based on the availability of pesticide application data. In chapter 1 we used standard telemetry techniques, which were used in studies on the common toad since the 80s (van Gelder et al., 1986; van Nuland and Claus, 1981). The daily relocation of individuals is quite time consuming, but the main downside is that each relocation produces only one data point. Thus, movements between two observations are calculated as a straight line, ignoring the actual movement path. Movements that end in the same hiding place as before might be overlooked completely, which might lead to an underestimation of the activity and of the actual exposure risk. To counter these limitations and to get more detailed data on the migration behaviour of amphibians, new technologies and methods will help in future studies. Initiatives like ICARUS (International Cooperation for Animal Research Using Space) aim to provide tracking devices that are light enough to be used for small vertebrate species, that have a high durability due to energy harvesting and that allow continuous tracking in almost real-time based on GPS and a receiver in space (Curry, 2018; Wikelski et al., 2007). Wireless biologging networks with tag masses between 1 and 2 g allow for a high spatial and temporal resolution tracking even in closed habitats (Ripperger et al., 2020) and 3D accelerometers allow for the collection of detailed behaviour and movement data and even for the estimation of the energy expenditure of an individual (Halsey and White, 2010). In addition, there is a recent trend to use detection dogs to find amphibians in the terrestrial habitat (Grimm-Seyfarth and Harms, 2019). This also includes newts (Grimm-Seyfarth and Harms, 2019) which are hardly considered in tracking studies and where data on habitat use is urgently needed (Denoël et al., 2013).

When data on habitat use and/or data pesticide applications are not available, different proxies for pesticide exposure can be used in future studies. The easiest way to reveal a previous exposure is to detect pesticides in an individual. However, this usually requires its euthanization for tissue sampling, which might limit the applicability of the method due to animal welfare or might restrict the study design. As an at least not lethal but still invasive alternative, silicon passive sampling devices can be attached to the skin of amphibians to measure pesticide exposure. Currently, this method is still limited to short study periods because sampling devices get lost after 1-2 weeks (Yaw et al., 2017). Instead of measuring the pesticides directly also enzymatic biomarkers that are linked e.g. to the detoxification can be analysed. Most sampling protocols are also invasive as blood or tissue is needed, but for common wall lizards (*Podarcis muralis*) buccal swabbing has successfully been tested as a non-invasive alternative (Mingo et al., 2019, 2016). Adapting this approach to amphibians might be promising in future studies, especially when data on enzymatic activities are combined with spatial data on the habitat use (i.e. tracking data) of an individual. In a recent study, Renoirt et al. (2021) managed to use stable isotopes to assess agricultural habitat use of the spined toad (*Bufo spinosus*).

With the arena choice experiment in which individuals were filmed over 24 h (chapter 2), I tested a novel, simple and inexpensive setup to study the avoidance behaviour of amphibians. However, our study raised several questions that should be addressed in the future. For example, it would be interesting if individuals from uncontaminated source populations show the same avoidance behaviour as the metamorphs from a contaminated pond surrounded by vineyards as in our study. Future tests should also focus on more realistic scenarios, such as the use of more natural soil with a higher content of organic matter that had not been oversprayed directly before the test. Also testing realistic application sequences with mixtures of multiple pesticides instead of single formulations would be interesting. Finally, field studies are needed to see if results from the laboratory can be transferred to the real world and thus if avoidance of pesticides leads to chemical landscape fragmentation.

To answer the question, which effects chemical landscape fragmentation has on a amphibian metapopulation, large scale landscape genetic studies are needed in study areas that consist of a mosaic of agriculturally used and unused patches. Thereby it is crucial to know the management type of the agricultural fields and especially the pesticide application schemes.

6. Conclusion

The studies presented in this thesis contribute to the knowledge of the situation of amphibians in agricultural landscapes, provide new insight into topics hardly investigated before and even come up with new methodological approaches for future studies. I could show that common toads use vineyards as habitat, but that they tend to avoid them (chapter 1), and that some pesticides frequently used in vineyards are avoided in a laboratory setup (chapter 2). Further, I found evidence that viticulture leads to a fragmentation of the landscape (chapter 3) and that pesticides used in viticulture might result in impairments of the reproduction capacity of amphibians (chapter 4). However, I could also show that some agricultural ponds can be suitable habitats for some species, although the quality of the aquatic and terrestrial habitat for early life stages should be increased (chapter 5). Despite finding hints for chemical landscape fragmentation in the laboratory, I was not yet able to provide a conclusive proof of this concept in the field. All presented studies are case studies focusing on single aspects of single species in a narrow ecological context. However, I see our results as pieces of a jigsaw puzzle, and together with dozens of other studies, they add up to a picture that is more and more complete. To protect amphibians, we must find a way back to heterogeneous agricultural landscapes that work without, or at least with a drastically reduced amount of (synthetic) pesticides. In the end, not only amphibians will profit from extensive agricultural landscapes, which are mosaics of different habitats with green corridors, but a whole range of species groups such as birds (Benton et al., 2002; Donald et al., 2006), bats (Froidevaux et al., 2017) or insects (Benton et al., 2002). Thus, conservation measures aiming at amphibians might bring us one step closer to the goals of the EU Biodiversity Strategy for 2030 (European Union, 2020), which emphasizes that “we need nature in our lives”.

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Declaration

I hereby declare that I independently wrote this thesis with the title “**Amphibians in a fragmented viticultural landscape**”. All used resources and references are specified in this work. Contributions of coworkers and coauthors have been clearly identified.

I did not use the assistance of a doctoral consultant (or a similar person) in return for payment.

I did not, and have never, submit this PhD thesis in identical or similar form elsewhere for a scientific examination.

I am aware that a violation of the above mentioned points can lead to a withdrawal of the doctoral degree and additional legal consequences.

Mödling (Austria),

Place, Date Christoph Leeb

Authors’ contribution

Chapter 1:

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Curriculum Vitae

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