



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 exist 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.

Key words. 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 temper-

ate 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 BEI-NUM 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* (RAZOU-MOWSKY, 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 Palatine 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, WTW, 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.

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 phenolo-

gy 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 limb 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 (Soft-Genetics). 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 conse-

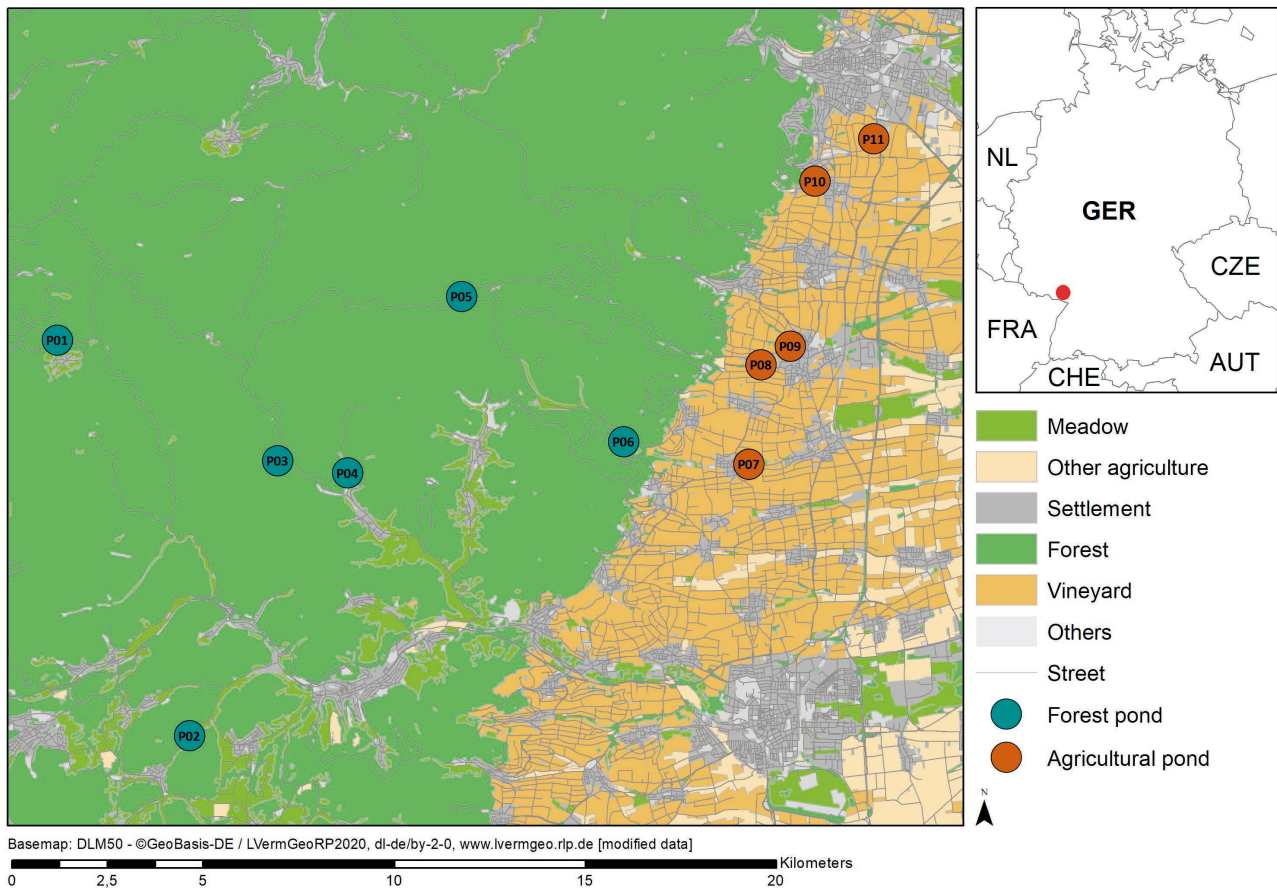


Figure 1. Map of study area with all sampled forest (n = 6) and agricultural ponds (n = 5).

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.

Pond-ID	Classification	genetic analysis	Number individuals used for					
			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	

quently 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 “divRstity” (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™ (JUNG) and stained with 0.5% cresylviolet (SINSCH et al. 2018). The midsection of the bone (diaphysis) was cross-sectioned at 12 μ m 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.

Statistical analyses

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

$$SVL \sim \text{pond type} + \text{meadow} + \text{submersed vegetation} + \text{sex} + (1|\text{pond-ID}).$$

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 $\Delta AICc < 4$ to the best model to have em-

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.

Pond-ID	Classification	Coordinates		Altitude (m)	Pond parameters				Land use in 400 m					Streets (m)
		x	y		pH	Conductivity ($\mu\text{S}/\text{cm}$)	Submersed vegetation (%)	Forest (%)	Agriculture Total (%)	Vineyard (%)	Settlement and Industry (%)	Meadow (%)	Other landuse (%)	
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	1	23	not	0
p				0.004	0.004	0.004	0.409	0.008	0.004	tested	0.013	0.151	tested	0.004

pirical 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.

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.

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 $\Delta AICc < 4$ (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 vegetation + 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

Table 3 continued

Response	Variable	Estimate	SE	lower CI 95%	upper CI 95%	z	p
Full model: LMM (T ~ Pond type + SVL + Pond type : SVL + SMI + Submersed vegetation + Meadow + (1 Pond-ID))							
T (n = 472)	Intercept	-0.653	0.894	-2.410	1.103	0.729	0.466
	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 (n = 74)	Intercept	-3.031	2.705	-8.429	2.367	1.101	0.271
	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 (n = 468)	Intercept	-3.597	1.376	-6.300	-0.894	2.608	0.009
	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 (n = 75)	Intercept	-0.453	4.933	-10.166	9.259	0.091	0.927
	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

Sampling and measured traits

A total of 923 individuals from six forest ponds (mean $n = 70.3 \pm 12.8$) and five agricultural ponds (mean $n = 100.2 \pm 50.1$) 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 $n = 38.0 \pm 2.2$). 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 $\Delta AICc < 4$ (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 Po5 the CF was significant longer and T was significant higher in March, while in the pond Po3 T was higher in May and no difference was observed in CF. For

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	

both traits, higher values were observed in Po7 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 new 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.

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 for-

est. 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

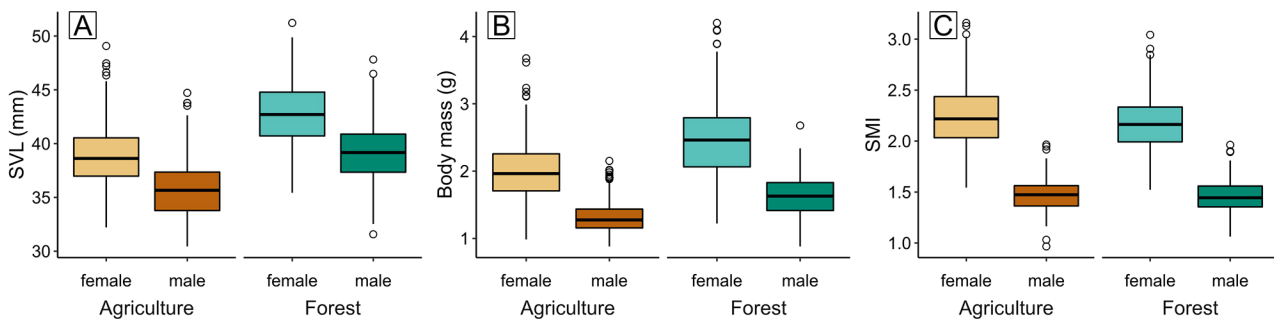


Figure 2. Boxplots of the snout-vent length (SVL; A), the body mass (B) and the scaled mass index (SMI; C) of male and female *Lissotriton helveticus* reproducing in forest (n = 6) and agricultural (n = 5) ponds. In each boxplot, the boundaries of the box are the 25th and 75th percentiles and the whiskers 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.

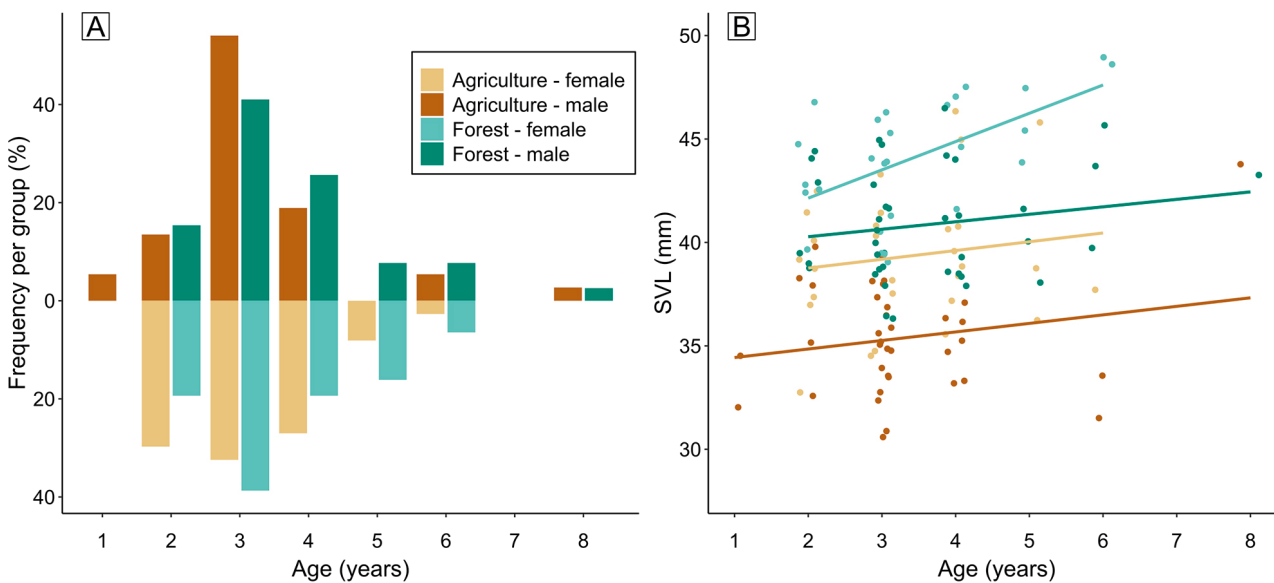


Figure 3. Demographic structure of *Lissotriton helveticus* captured in forest and agricultural ponds (A) and correlation between snout-vent length (SVL) and age (B). No difference in the median age of males and females reproducing in forest and agricultural ponds, respectively, was found. The age had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

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 (F_{IS}) 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	0.5	-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

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

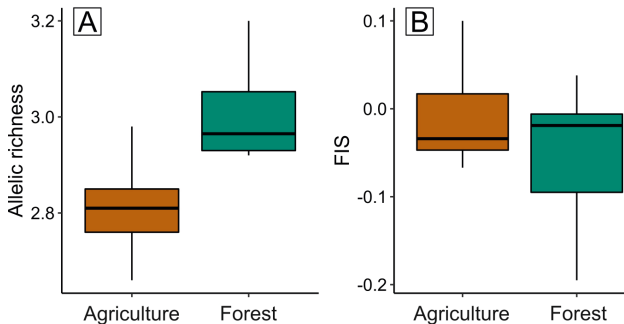


Figure 4. Boxplots showing the allelic richness (A) and the inbreeding coefficient F_{IS} (B) of *Lissotriton helveticus* populations situated in the forest ($n = 6$) and the agriculture ($n = 5$). In each boxplot, the boundaries of the box are the 25th and 75th percentiles and the whiskers correspondent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

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 (LEE B et al. 2020a) and that there is an avoidance behaviour against some pesticides frequently used in viticulture (LEE B 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 $F_{IS} = 0.251$; ISSELLIN-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, agricultural 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, conserva-

tion 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.

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Supplementary data

The following data are available online:

Supplementary document 1. Plot of the first and second principal component (PC).

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

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

Supplementary document 4. Length of the caudal filament and max. height of the tail of male *Lissotriton helveticus* captured in March/April and May 2018 and the results of parametric and nonparametric tests.

Supplementary document 5. List of all full and candidate models that were used to calculate model-averaged coefficients describing the response variables with the model type, degrees of freedom, the Akaike Information Criterion with a correction for small sample sizes, the difference to the best model and the weight of a candidate model.

Supplementary document 6. Results from testing for linkage disequilibrium for each locus pair across all populations with GENEPop 4.6 (Fisher's method).

Supplementary document 7. Results from testing each population and Loci for Hardy-Weinberg equilibrium.