Hybridization between pond turtles (*Emys orbicularis*) subspecies in natural and human-mediated contact zones

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SUMMARY

Hybridization is particularly important for evolutionary and speciation processes. It promotes evolutionary divergence between lineages by reinforcing reproductive isolation mechanisms to avoid the erosion of the spatial genetic structure and the production of unfit hybrids. Additionally, hybridization creates novel taxa by the fusion of interacting lineages. However, introgressive hybridization leads to conservation problems when introgression happens between threatened lineages and non-native or domesticated lineages. In such cases, introgressive hybridization may lead to the genetic swamping of one lineage and to the loss of the entire taxon. Hybrids often harbour novel genetic combinations and novel adaptations that provide them a higher potential to adapt to environmental changes and colonize new ecological niches. In the future, conservation of hybrids might provide additional genetic diversity for species to cope with alterations in their environment. In my thesis, I investigated hybridization and introgression between European pond turtle subspecies (*Emys orbicularis*) by combining nuclear microsatellites and mitochondrial cytochrome *b* gene: I reported that introgressive hybridization between pond turtle subspecies occured in natural and human-mediated contact zones. Outcomes of introgressive hybridization between European pond turtle (*Emys orbicularis*) subspecies indicated that *E. o. orbicularis* and *E. o. hellenica* subspecies and hybrids may have better fitness than *E. o. galloitalica* subspecies and hybrids under Swiss environmental conditions. Furthermore, I developed an eDNA-based methodology to detect the presence of pond turtles and investigated the efficiency of two water sampling methods. Finally, results of this thesis provided scientific knowledge to the Swiss national conservation programme of the European pond turtle.

GENERAL INTRODUCTION

Evolutionary divergence between geographically isolated populations is central to the formation of new species and to conspecific genetic differentiation (Coyne 1992). In allopatric speciation, previously interfertile species become isolated by geographic barriers and evolve towards genetically, temporally or behaviourally distinct taxa. Additionally, reproductive isolation mechanisms may avoid the production of viable and fertile offspring, thus reinforcing isolation and promoting speciation processes. Speciation processes may then evolve towards distinct lineages, unable to hybridize and inapt to produce viable offspring. In other cases, lineages may exhibit various stages of speciation processes, giving rise to debates about taxonomic units and species definitions (De Queiroz 2007).

Evolution and speciation are highly dynamic processes in contact zones between divergent lineages (Mallet 2007; Genovart 2009; Abbot et al. 2013). Environmental conditions, mate recognition preferences, population densities, reproductive barriers and natural selection lead to tensions in contact zones, where a balance occurs between dispersal of lineages and selection against hybrid offspring. Indeed, natural selection may favour hybrid individuals with intermediate phenotypes in intermediate habitats while it may select against hybrid individuals in parental habitats. Together, hybridization and natural selection generate individuals with high genetic and phenotypic diversities, able to cope with new environmental conditions and to colonize new ecological niches (Dowling and Secor 1997; Dittrich-Reed and Fitzpatrick 2013).

Divergent taxa in hybrid zones have evolved to cope with hybrid offspring or have developed mechanisms to select against maladaptive mating between divergent lineages (Edmands 2002). In the absence of reproductive isolation mechanisms, repeated hybridization may lead to the introgression of lineages (Servedio and Noor 2003; Crispo et al. 2011; Abbott et al. 2013). As Human activities increasingly alter the integrity of geographic barriers between lineages by disturbing ecosystems and by translocating individuals into new locations, previously allopatric taxa may meet in secondary human-mediated contact zones (Allendorf et al. 2001; Hobbs et al. 2009). Consequently, natural patterns of hybridization are altered and outcomes of recent introgression between previously allopatric taxa are difficult to predict.

Introgressive hybridization between lineages have different fitness outcomes for hybrid offspring, depending on genetic divergences between parental lineages, their reproductive compatibility and relative fitness of each parental taxon (Edmands 2002; Rhode and Cruzan

2005). Furthermore, it may lead to the erosion of spatial genetic structure between lineages and to the genetic swamping of taxa. Additionally, by disrupting coadapted genes complexes, introgressive hybridization may result in the loss of local adaptations (Arnold 1992). In such situations, hybrid offspring often exhibit lower fitness than parental taxa. However, when hybrid offspring exhibit novel genetic combinations and heterosis, they may outperform parental lineages (Barton 2001; Burke and Arnold 2001), or when they are able to take advantage from new environmental conditions and ecological niches that were not previously occupied by the native parental genotypes (Lewontin and Birch 1966; Dittrich-Reed and Fitzpatrick 2013).

Hybridization events are widely distributed among highly diverging animal and among reptiles in particular (see Jancuchova-Laskova et al. 2015 for a review). Interspecific hybridization has been reported in turtles (Karl et al. 1995; Stuart and Parham 2007) and tortoises (Garrick et al. 2014; Edwards et al. 2016) with introgression events between lineages (Vilaça et al. 2012; Garrick et al. 2014; Edwards et al. 2016).

Hybrids may be of particular interest in a conservation perspective, as they may introduce additional genetic diversity to cope with alterations to ecosystems, especially in the setting of climate change. On the contrary, hybridization between lineages may represent a threat when occurring between rare and common taxa, or between wild and domesticated taxa. In these cases, hybridization may lead to the loss of local adaptations (ecotypes) or taxa by repeated introgression (Gottelli et al. 1994; Randi 2008).

Scientific research is often difficult to implement in aquatic ecosystems, where traditional investigation methods like capture sessions may be hard to set up. The development of new scientific research techniques may be particularly effective for population genetic studies in aquatic ecosystems. In the future, genetic and demographic studies may involve new tools like the use of drones or next-generation sequencing techniques to investigate areas with difficult accesses like aquatic ecosystems.

Focus on the thesis

The aims of this thesis were to investigate hybridization patterns between European pond turtle (*Emys orbicularis*) subspecies in contact zones, to evaluate fitness consequences of introgressive hybridization between subspecies and to develop a new methodology for turtle population genetic studies.

During Pliocene and Pleistocene events, genetic drift and the absence of gene flow between glacial refugia fostered the genetic differentiation of many taxa: during the Pliocene, the disparition of the Parathethys Sea and the apparition of marked seasons have presumably promoted the adaptive radiation of many taxa, like the European pond turtle (Fritz 2003). During this period, the European pond turtle evolved into genetically distinct mitochondrial haploclades (I; III to X). During the Pleistocene climatic oscillations, species retreated into glacial refugia along the Mediterranean Sea coasts, in Balkans, in Anatolia and in the Caucasus. In these glacial refugia, the European pond turtle further differentiated and haploclade I gave the offshoot haploclade II (Lenk et al. 1999; Fritz et al. 2005, 2007).

These distinct mitochondrial haploclades correspond to morphologically, behaviourally and genetically defined subspecies (Fritz 2003). During the Holocene recolonization of Europe from their glacial refugia, distinct pond turtle subspecies expanded northwards. Thanks to rivers and aquatic networks, this species rapidly dispersed and colonized new regions (Sommer et al. 2007, 2009). Nowadays, distinct subspecies are widely distributed from North Africa over a large part of Europe to Asia Minor (Fritz 2003; Fritz et al. 2005): pond turtles with haploclades belonging to the subspecies *E. o. orbicularis* are currently found in the Danube and Oder rivers catchment basins, in the Balkan Peninsula, Southern France and Northern Spain. Turtles with haploclades IV belong to the subspecies *E. o. hellenica* and have a circumadriatic distribution. Both subspecies live in marshlands and in oxbow lakes with aquatic vegetation. On the contrary, turtles with haploclades V belong to the subspecies *E. o. galloitalica* and are distributed on the Western Apennine peninsula, in Sardinia, in Corsica and in Southern France. This latter subspecies is mainly reported in mountain stream waters (Lenk et al. 1999; Fritz et al. 2005). In Switzerland, only *E. o. orbicularis* and *E. o. hellenica* are considered as native subspecies northern and southern of the Alps, respectively. Nowadays, distinct subspecies with different haploclades meet in natural contact zones like Southern France or Eastern Europe (Lenk et al. 1999). However, hybridization events and outcomes have not been studied in such contact zones. In **Chapter 1**, I investigated

hybridization patterns between pond turtle subspecies in a natural contact zone located in Southern France by combining nuclear and mitochondrial markers. I further compared introgression patterns displayed by mitochondrial and nuclear genetic markers.

As Human activities have created new secondary-contact zones between pond turtle subspecies by translocating individuals in Europe, contact between native and non-native pond turtle subspecies that do not meet naturally in contact zones may lead to hybridization and introgression events. However, little is known about human-mediated hybridization events in pond turtles. In **Chapter 2**, I investigated human-mediated hybridization patterns between pond turtles subspecies and their consequences on the fitness in a complex population with three subspecies: due to human introductions in the canton of Geneva, a breeding population had been created since the 1950s after the canalization of the Rhône River and the creation of an oxbow lake. The second aspect of my thesis was therefore to reveal its genetic composition by investigating hybridization between *E. orbicularis* subspecies. Furthermore, relative survival and growth rates were compared between hybrid and non-hybrid individuals to evaluate if "unnatural" hybridization between subspecies might have negative consequences on hybrids fitness.

Aquatic turtle populations are generally monitored with time-consuming and environment-disturbing techniques. Indeed, visual observations and monitoring sessions with boats and nets are often difficult to set up and prone to false-negative detection rates. As a consequence, new tools to detect species in sensitive ecosystems need be developed in order to improve the detectability of pond turtles in aquatic ecosystems. Environmental DNA (eDNA) is currently considered as a rapid and cost-effective tool to manage and detect the presence of elusive (Wilcox et al. 2013; Sigsgaard et al. 2015) and invasive species (Ficetola et al. 2008; Jerde et al. 2011; Piaggio et al. 2014). Additionally, it is assumed successful in investigating quantitative relationships between biomass and the relative abundance of eDNA detected in the environment (Takahara et al. 2012; Thomsen et al. 2012). However, the applicability of eDNA to low secreting taxa like reptiles remains highly unknown. Therefore, the third aspect of my thesis was to develop an eDNA-based methodology for genetic studies on turtles and to evaluate its reliability to reptiles in aquatic ecosystems (**Chapter 3**). The potential of the eDNA-based methodology to detect the presence of the European pond turtle was investigated in environmental water samples from artificial and natural ponds. Moreover, two different water sample collection methods (filtration and precipitation) were compared to reveal the

most appropriate method to pursue genetic studies on turtles in ponds. Finally, I investigated the reliability of eDNA to evaluate the relative abundance of turtles in ponds.

Finally, the last aspect of my thesis was to provide scientific support for the conservation programme of the European pond turtle by monitoring the first individuals released officially in the canton of Geneva, by analysing genetically suitable turtles for reintroduction and reinforcement events and by providing both field and genetic recommendations for the conservation programme of the species (**Chapter 4**).

CHAPTER 1

Hybridization between turtle subspecies: a case study with the European pond turtle (*Emys orbicularis*)

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RESEARCH ARTICLE

Hybridisation between turtle subspecies: a case study with the European pond turtle (*Emys orbicularis*)

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Abstract Genetic introgression has recently become an important concern for conservation genetics as it can occur between rare and related common species, between various genetic groups and between individuals from different origins. Our aims were: (i) to determine whether hybridisation or introgression occurs between *Emys orbicularis* subspecies in a natural contact zone in France; (ii) to determine the geographic extent of the contact zone between distinct subspecies in France; (iii) to evaluate at which level introgression occurs, and finally; (iv) to evaluate whether combining mitochondrial and nuclear genetic markers reflects the same contact zone as when only one genetic marker is used. Introgression was evaluated by genotyping biparentally inherited microsatellites and sequencing the maternally inherited cytochrome *b* gene of French populations. We demonstrated strong introgression between subspecies under natural conditions in the old contact zone in southern and eastern France. Our results corroborated that introgression reflects past natural events, but also demonstrated that human impact has altered these patterns. We finally confirmed that the combination of mitochondrial and nuclear genetic markers is more appropriate to reveal introgression than the use of only one genetic marker.

Keywords Conservation genetics · France · Hybridisation · Introgression

Introduction

Thermophilic species survived the last glaciation in southern refugia, where climatic conditions were suitable, such as the southern European Peninsulas (Taberlet et al. 1998; Hewitt 1999). Geographic barriers—for instance seas and mountains—fragmented populations and induced genetic differentiation into isolated glacial refugia (Hewitt 1996). During the Holocene, many taxa with genetically distinct lineages recolonised Europe and met in contact zones, which occasionally led to hybridisation between lineages (Hewitt 1999). For instance, southern and eastern France represent contact zones for many taxa, including the European Pond Turtle, *Emys orbicularis*. The complex postglacial recolonisation history of this species, in which several distinct genetic lineages from southern refugia are involved, makes it a particularly interesting species to investigate hybridisation in natural contact zones (Pedall et al. 2011; Vamberger et al. 2015). Although the species was considered monotypic for decades (Boulenger 1889; Ernst and Barbour 1989), it has been shown to be one of the genetically most fragmented vertebrates of the Western Palaearctic (Lenk et al. 1999; Fritz et al. 2009). Pliocene and Pleistocene vicariance events have induced the radiation of *E. orbicularis*. During glacial periods, *E.*

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orbicularis was mainly confined to refugia along the Mediterranean Sea coasts, in the Iberian and Italian peninsulas, in the southern Balkans, Anatolia, the Caucasus and along the south coast of the Caspian Sea, where genetic drift and the absence of gene flow fostered the genetic differentiation of distinct mitochondrial haploclades, which correspond to the morphologically and genetically defined subspecies (Lenk et al. 1999; Fritz et al. 2004, 2005a, b, 2007, 2009). During the Holocene, turtles from distinct refugia expanded northwards: *Emys orbicularis orbicularis* (harbouring haploclade II) recolonised Western and Central Europe from a Balkan refuge, while the range expansions of *Emys orbicularis hellenica* (haploclade IV) from a south-eastern Italian refuge and *Emys orbicularis galloitalica* (V) from a south-western Italian refuge were blocked by the Alps in the north (Lenk et al. 1999; Sommer et al. 2009). *E o. orbicularis* and *E. o. galloitalica* are currently considered to be native subspecies in France, the first being distributed mainly in central and western France and the second, in southern France (Fritz et al. 2005a, 2007).

It can be assumed that the natural distribution range of these subspecies has been impacted by human activity. It is well known that humans alter historical, physiogeographic and climatic barriers, and thereby, affect the natural contact zones of taxa. Therefore, previously isolated taxa might meet and hybridise in novel anthropogenic contact zones (Hobbs et al. 2009). As a consequence, hybridisation has become an important concern for conservation genetics, particularly since the discovery of rare species introgressed by related common ones and the loss of local adaptations (ecotypes) by repeated introgression into rare species, subspecies or populations (Gottelli et al. 1994; Randi 2008). The century-long trade in European pond turtles for food purposes and later for the pet trade (Dahms 1912; Kinzelbach 1988; Schneeweiss 1997) not only drove local populations to extinction, but also led to the introduction of turtles outside their native distribution ranges (Fritz et al. 2004; Velo-Antón et al. 2011; Vamberger et al. 2015).

In this study, we investigated the introgression patterns in the natural contact zone of two pond turtle subspecies in France. Using nuclear and mitochondrial markers, our aims were: (i) to determine the exact location and size of the contact zone; (ii) to assess whether introgression occurs and if so, (iii) to what extent; and finally (iv) to evaluate whether mitochondrial and nuclear genetic markers display the same introgression patterns. Finally, our results are discussed in the context of ongoing reintroduction programmes in Europe.

Through the use of biparentally inherited markers, we expected to detect gene flow between both native subspecies in the contact zone ranging from southern to eastern France. We anticipated a more complex introgression pattern in the Camargue region, because the Rhône river might allow gene flow from northern populations (*E. o. orbicularis*) to southern populations (*E. o. orbicularis* and *E. o. galloitalica*). As central and western populations are geographically distant from *E. o. galloitalica* populations, no introgression is expected there. Similarly, as eastern (Provence) and southern populations in the Rhône estuary region belong to two distinct hydrographic units, no introgression is expected for eastern populations. As a consequence, we presumed that central, western and eastern populations should possess a lower genetic variability than southern populations.

Materials and methods

Genetic analyses

Eighteen populations with a total of 304 wild turtles were sampled between 2002 and 2012. Total genomic DNA was extracted from blood samples, buccal swabs and claws after an initial incubation at 56 °C in ATL lysis buffer (Qiagen, Hombrechtikon, Switzerland) for 4, 12 and 48 h respectively (with the addition of 20 µL Proteinase K (Qiagen) after 24 h to improve DNA digestion from claws). DNA extraction followed the DNeasy Blood and Tissue Handbook manufacturer's handbook. Concentration of DNA was estimated with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, US).

PCR reactions were performed in 25-µL volumes with 2 µL DNA template, 8 µL sterile dH_2O, 12.5 µL MasterMix (MasterMix Kit, Qiagen) and 1.3 µL of each primer in an Eppendorf thermocycler (Eppendorf, Wesseling-Berzdorf, Germany). The cytochrome *b* gene (cyt *b*) was amplified using the primers mt-A and H15909 (Lenk and Wink 1997; Lenk et al. 1999). The seven microsatellite loci, msEo2, msEo29, msEo41 (Pedall et al. 2009) and GmuB08, GmuD51, GmuD87 and GmuD114 (King and Julian 2004) were amplified following the conditions mentioned in the respective publications, with forward primers labelled with fluorescent dye. The cyt *b* sequences were analysed by Macrogen (Amsterdam, the Netherlands) on an ABI 3730XL sequencer (LifeTechnologies, Switzerland) and compared to reference sequences from GenBank (Accession numbers AJ131407-AJ131426; AM269887-AM269893; AY652865-AY652889; GU645 999-GU646001), to assign them to a previously identified haploclade following Lenk et al. (1999), Fritz et al. (2005b, 2007) and Velo-Antón et al. (2011). Microsatellite PCR products were analysed on an ABI 3130 sequencer

(LifeTechnologies, Luzern, Switzerland) and allele sizes were scored using the program PEAKSCANNER 1.0 (LifeTechnologies, Luzern, Switzerland).

Statistical analyses

The occurrence of null alleles in microsatellite markers was assessed using MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). Hardy–Weinberg disequilibrium, private alleles, observed (Ho) and expected (He) heterozygosities were calculated with GENALEX 6.5.1 (Peakall and Smouse 2012) and tested with Chi squared tests. Allelic richness (Ar) was calculated for each locus and linkage disequilibrium between loci were calculated and tested using FSTAT 2.9.3 (Goudet 1995) based on three diploid individuals and 10,000 bootstraps. *F*-statistics were estimated to determine the extent of deviation from random mating within populations (F_{IS}) and to measure genetic divergence between populations (F_{ST}; Wright 1921; Frankham et al. 2002) using the software FSTAT; the significance level was evaluated by 10,000 randomisations. Putative hybrids were detected with STRUCTURE 2.3.4 (Pritchard et al. 2000) using the admixture model with the correlated alleles setting. The number of mitochondrial haploclades was set as a putative cluster number and the likelihood values for $K = 1$–6 were estimated for 10 runs each, with 1.5×10^6 MCMC repetitions using the first 0.5×10^6 as burn-in. The most probable number of clusters was then assessed using the ΔK method (Evanno et al. 2005). Turtles with a probability of assignment $P_{assign} > 90\%$ were considered as 'pure', whereas individuals with $P_{assign} < 90\%$ were considered as recently introgressed (Vähä and Primmer 2006). In the face that microsatellite markers indicate only for a few generations admixture (Sanz et al. 2009), turtles were treated as old introgressed when they clustered to one cluster with a probability of assignment $P_{assign} > 90\%$ but contained the haploclade typical for the other cluster.

As we expected that some populations had undergone a strong reduction in population size, populations with ten individuals or more were investigated using BOTTLENECK 1.2.02 (Cornuet and Luikart 1996), with the two-phase model (TPM, combining 95% of SSM and 5% of IAM) and a variance value of 12, and were tested with Wilcoxon tests as suggested in Piry et al. (1999).

In southern populations (Aigues-Mortes, Pont-de-Gau, Tour-du-Valat, Marais des Baux, Bolmon and Vigueirat), gene flow in both directions was examined using MIGRATE-n 3.6.8 (Beerli and Palczewski 2010). After several simulation tests, a final run was conducted with the parameters mentioned in Supplementary material Table S2.

Results

Statistical analyses

Analyses conducted with MICRO-CHECKER and FSTAT neither detected null alleles for all populations nor significant linkage disequilibrium among all loci (Text S1). Consequently, all microsatellites were used for further analyses. The number of analysed turtles per population, allelic richness, private alleles and averaged observed and expected heterozygosities are shown in Table 1.

Genetic diversity was similar among populations except for the Chauzon population, which displayed a limited observed and expected heterozygosity and a significantly lower allelic richness than all other populations (ANOVA, $F = 19.106$, $p < 0.001$). This population was particularly small and isolated (the size was estimated to about 20 individuals, M. Cheylan unpublished data). However, no bottleneck and no private alleles were detected for this population (Table 1).

Several populations possessed one or more loci that were not in Hardy–Weinberg equilibrium. However, most populations with a strong Hardy–Weinberg disequilibrium were located in southern and eastern France (Table 1), where a contact zone between both native subspecies was previously reported (Lenk et al. 1999; Fritz et al. 2005a) and in a contact zone with recent admixture, no Hardy–Weinberg equilibrium is expected.

Low F_{ST} values ($-0.005 < F_{ST} < 0.111$) were found between geographically close populations. However, in contrast, high F_{ST} values were observed between the Chauzon population and all other populations ($0.254 < F_{ST} < 0.349$), what may be due to the very limited genetic diversity within this population (Table S1).

Subspecies and contact zones

From 303 analysed turtles, 180 (60%) contained haplotypes of the subspecies *E. o. orbicularis*, 111 turtles (36%) haplotypes of *E. o. galloitalica* and 12 turtles haplotypes of the non-native *E. o. hellenica* (Table 1; Fig. 1a). In central and western populations, only haplotypes of *E. o. orbicularis* were found, whereas in eastern and southern populations, haplotypes of *E. o. galloitalica* and *E. o. orbicularis* were discovered, confirming the contact zone identified by Fritz et al. (2005a). In addition, 12 turtles (4%) with haplotypes of *E. o. hellenica* were recorded in Aigues-Mortes (11 individuals) and Pont-de-Gau (one individual).

The ΔK method suggested two as the most likely number of clusters using microsatellite loci (Fig. S1b). Individuals of the first cluster with an assignment

Table 1 Genetic diversity, mitochondrial haplotypes and proportion of introgression for each population following the STRUCTURE analyses of nuclear genes

Regions	Populations	N individuals	N loci not at the HW equilibrium	Allelic richness for each pop.	N private alleles	Av. Ho	Av. Hs	Fis all	Bottleneck	mtDNA orbicularis	mtDNA galloitalica	mtDNA hellenica
Central	Brenne	35	2	3.50	2	0.722	0.729	0.026	No	35	0	0
Western	Jemay	16	3	3.17	11	0.504	0.610	0.203	No	16	0	0
	Haillan	19	1	3.98	4	0.749	0.784	0.071	No	19	0	0
	Graveyron	9	0	4.04	0	0.772	0.755	0.038	[a]	9	0	0
	Bassussarry	4	0	3.51	1	0.752	0.658	−0.009	[a]	4	0	0
	Urt	5	0	3.69	0	0.800	0.689	−0.054	[a]	5	0	0
	Dax	6	0	3.79	0	0.719	0.706	0.122	[a]	6	0	0
	Labenne	8	0	4.08	2	0.786	0.753	0.025	[a]	8	0	0
	All pop	67	4	3.73	–	–	–	–	–	67	0	0
Southern	Aigues-Mortes	21	0	3.82	2	0.789	0.776	0.009	No	3	7	11
	Pont-de-Gau	34	1	3.60	2	0.649	0.732	0.129	No	29	4	1
	Tour-du-Valat	30	3	3.49	2	0.652	0.728	0.122	No	16	14	0
	Vigueirat	12	2	3.87	1	0.774	0.751	0.012	No	0	12	0
	Marais des Baux	14	0	3.56	0	0.648	0.710	0.126	Yes	2	12	0
	Bolmon	17	1	3.43	1	0.757	0.715	−0.027	No	2	15	0
	All pop	128	7	3.62	–	–	–	–	–	52	64	12
Eastern	Ramatuelle	7	1	3.65	2	0.588	0.633	0.148	[a]	0	7	0
	St-Tropez	6	1	3.75	1	0.690	0.676	0.076	[a]	0	6	0
	Plan-de-la-Tour	50	2	3.50	8	0.676	0.711	0.075	No	16	34	0
	All pop	63	4	3.64	–	–	–	–	–	16	47	0
Ardèche	Chauzon	10	0	2.08	0	0.443	0.384	−0.134	No	10	0	0

Table 1 continued

Regions	Populations	N ind. assigned to cluster 1 (prob. of assignment >90%)	% pop. assigned to cluster 1	N ind. assigned to cluster 2 (prob. of assign. >90%)	% pop. assigned to cluster 2	Introgressed turtles						
						N putative recent hybrids (probability of assign. <90%)	% ind. assigned to putative recent hybrids	N putative old hybrids with mtDNA orbicularis assigned to cluster 2 (prob. of assign. >90%)	N putative old hybrids with mtDNA galloitalica assigned to cluster 1 (prob. of assign. >90%)	N putative old hybrids with mtDNA hellenica assigned to clusters 1 or 2 (prob. of assign. >90%)	Total N ind. assigned to putative old hybrids	% ind. assigned to putative old hybrids
Central	Brenne	34	97	0	0	1	3	0	0	0	0	0
Western	Jemay	16	100	0	0	0	0	0	0	0	0	0
	Haillan	9	47	0	0	10	53	0	0	0	0	0
	Graveyron	5	56	1	11	3	33	1	0	0	1	11
	Bassussarry	2	50	0	0	2	50	0	0	0	0	0
	Urt	3	60	0	0	2	40	0	0	0	0	0
	Dax	3	50	1	17	2	33	1	0	0	1	17
	Labenne	5	63	0	0	3	37	0	0	0	0	0
	All pop	43	64	2	3	22	33	2	0	0	2	3
Southern	Aigues-Mortes	14	67	0	0	7	33	0	3	8	11	52
	Pont-de-Gau	27	79	0	0	7	21	0	3	1	4	12
	Tour-du-Valat	26	87	0	0	4	13	0	12	0	12	40
	Vigueirat	7	58	0	0	5	42	0	7	0	7	58
	Marais des Baux	6	43	2	14	6	43	1	6	0	7	50
	Bolmon	4	24	2	12	11	64	0	4	0	4	24
	All pop	84	66	4	3	40	31	1	35	9	45	35
Eastern	Ramatuelle	0	0	4	57	3	43	0	0	0	0	0
	St-Tropez	1	17	5	83	0	0	0	1	0	1	17
	Plan-de-la-Tour	0	0	48	96	2	4	16	0	0	16	32
	All pop	1	2	57	90	5	8	16	1	0	17	27
Ardèche	Chauzon	0	0	10	100	0	0	10	0	0	10	100

[a] Bottleneck analyses were not performed on these populations as they comprise less than ten individuals

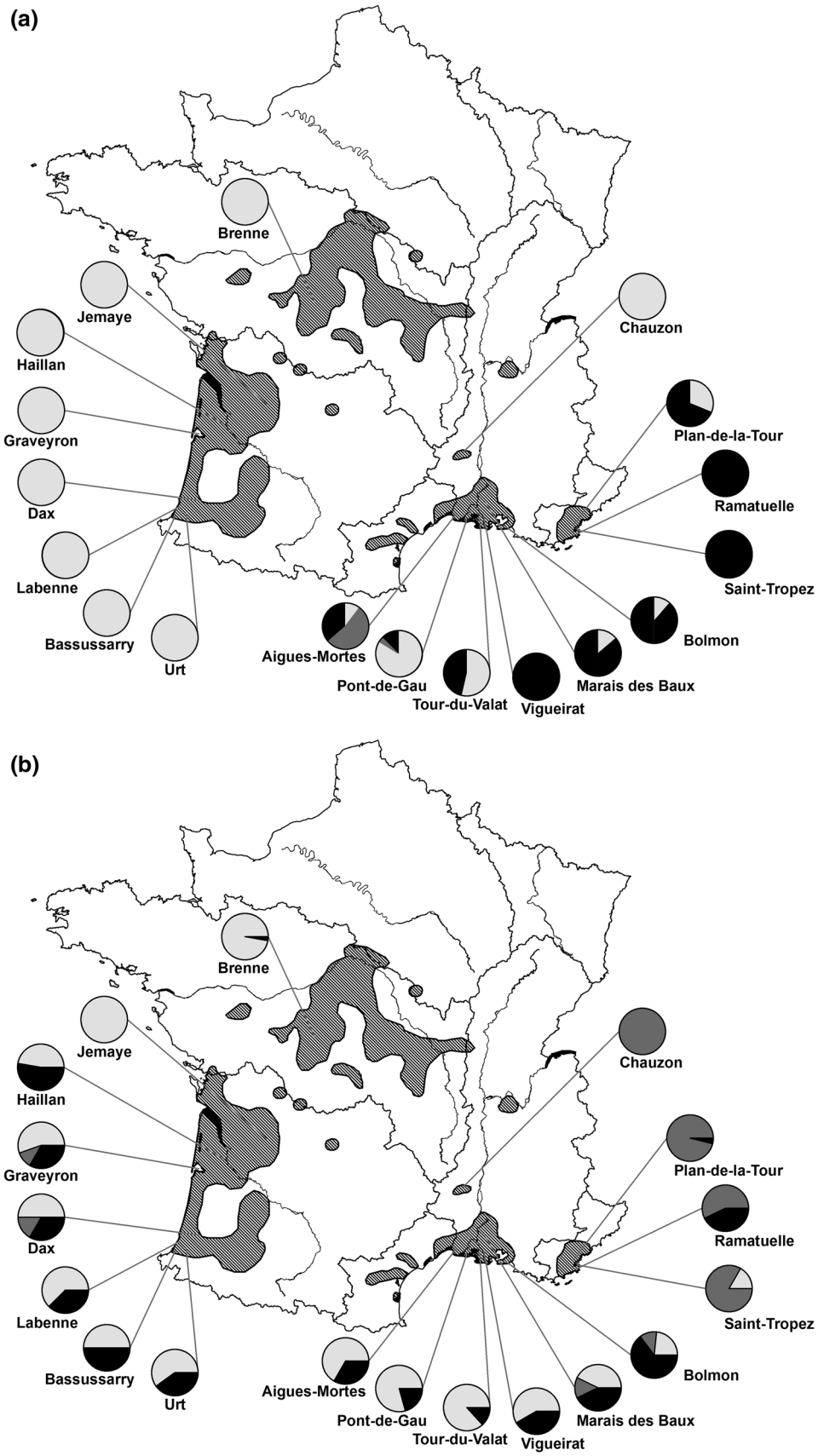

Fig. 1 Geographic extent of the contact zone between *Emys orbicularis* subspecies in France and levels of introgression in each population. Limits of catchment basins are indicated with a continuous line. **a** Proportions of individuals belonging to each subspecies determined by mitochondrial DNA: *E. o. orbicularis* are represented in *light grey*, *E. o. galloitalica* in *black*, and *E. o. hellenica* in *dark grey* (cf. Table 1); **b** proportions of introgressed and non-introgressed individuals determined by the STRUCTURE analyses based on nuclear DNA: 'pure' *E. o. orbicularis* are represented in *light grey*, 'pure' *E. o. galloitalica* in *dark grey* and admixed individuals in *black* (cf. Table 1)

probability greater than 90% represented mostly *E. o. orbicularis*, and individuals of the second cluster with an assignment probability greater than 90%, mostly *E. o. galloitalica*. Thus, the first cluster was assumed to match with non-introgressed 'pure' *E. o. orbicularis* (light grey in Fig. 1b), and the second, with non-introgressed 'pure' *E. o. galloitalica* (dark grey in Fig. 1b). Turtles assigned with lower assignment probabilities were considered to be of hybrid origin (black in Fig. 1b). Such admixed turtles were found in all populations except in one western population (Jemaye).

No third putative cluster corresponding to *E. o. hellenica* was detected. Individuals with the mitochondrial DNA of *E. o. hellenica* (a circum-Adriatic subspecies; Fritz et al. 2007; Vamberger et al. 2015) were grouped within cluster 1 (see Fig. S2). However, when STRUCTURE analyses were run within cluster 1 alone (results not shown), the Aigues-Mortes turtles were distinct.

Thus, our data provide evidence for large hybrid zones between *E. o. galloitalica* and *E. o. orbicularis* in southern and eastern France, with more than half of the turtles being considered as introgressed by one or the other subspecies (N = 107; 56%): one-quarter of the turtles were revealed as recent hybrids ($N_{\text{recent hybrids}} = 40$; 31% of the southern populations; $N_{\text{recent hybrids}} = 5$; 8% of the eastern populations), whereas one-third of the turtles were revealed as old introgressed individuals, i.e., individuals with nDNA and mtDNA assigned to different subspecies ($N_{\text{old hybrids}} = 45$; 35% of the southern populations; $N_{\text{old hybrids}} = 17$; 27%% of the eastern populations). In southern populations, an old introgression from *E. o. orbicularis* to *E. o. galloitalica* was found for 35 turtles harbouring haplotypes of *E. o galloitalica,* whereas old introgression into the opposite direction was revealed for only one turtle. On the contrary, in eastern populations, an old introgression from *E. o. orbicularis* into *E. o. galloitalica* was unravelled for only one individual harbouring a haplotype of *E. o. galloitalica*, whereas an old introgression from *E. o. galloitalica* into *E. o. orbicularis* was found for 16 individuals with haplotypes of *E. o. orbicularis* (Table 1).

Migration rates between populations in the contact zone are indicated in Table S2. Globally, the populations Tour-du-Valat and Pont-de-Gau (ordered according to their respective emigration level) were shown to be source populations, whereas Aigues-Mortes, Marais des Baux, Vigueirat and Bolmon could be considered as sink populations ($M_{\text{Pont-de-Gau+Tour-du-Valat} \rightarrow \text{Marais des Baux+Bolmon+Vigueirat+Aigues-Mortes}} = 1304.7$). Generally, there was a higher migration between the populations of Vigueirat, Marais des Baux and Bolmon (mainly *E. o. galloitalica*), compared to the migration between populations harbouring mainly haplotypes of *E. o. orbicularis* (Pont-de-Gau and Tour-du-Valat). However, extensive migration was also observed between these two groups (Table S2).

Discussion

The mitochondrial results are in agreement with previous studies about the phylogeography of the European pond turtle in France (Lenk et al. 1999; Fritz et al. 2005a, 2007; Pedall et al. 2011) and confirmed the occurrence of a contact zone between the subspecies *E. o. orbicularis* and *E. o. galloitalica* for southeastern France. However, the combination of mtDNA and nuclear markers demonstrated a more complex pattern of introgression between the two subspecies than previously recognized, underlining that highly variable nuclear markers are highly informative for studying contact zones.

In the contact zone, the use of nuclear markers allowed to attest extensive introgression between the two subspecies. There, a larger proportion of turtles was assigned to the first cluster (representing genotypically 'pure' *E. o. orbicularis*) and only a few individuals were assigned to the second cluster ('pure' *E. o. galloitalica*), suggesting pronounced eastwards directed nuclear introgression from *E. o. orbicularis* into *E. o. galloitalica*.

However, introgression patterns in the contact zone vary locally: several turtles from southern populations contain mtDNA of *E. o. galloitalica* (haploclade V), but are assigned to *E. o. orbicularis* by analyses of microsatellites, the majority of the eastern populations and the Chauzon population contain many turtles assigned to or related to *E. o. galloitalica* using microsatellites and approximately 50% (Plan-de-la-Tour) or all of these turtles (Chauzon) yield haploclade II, which is typical for *E. o. orbicularis.*

These observed mismatches between nDNA and mtDNA probably reflect not only different inheritance modes (microsatellite loci are biparentally inherited, in contrast to the purely maternal inheritance of mtDNA), but also colonization histories: if we consider that males are more prone to dispersal (Ficheux 2013), then our results suggest that the southern populations were first colonised by *E. o. orbicularis* expanding in the Holocene their range southwards through the Rhône valley (as indicated by mtDNA) and later were invaded by *E. o. galloitalica* (as indicated by the nuclear introgression) expanding their range westwards.

This hypothesis is in line with a glacial refugium used by *E. o. galloitalica* in the western Italian peninsula and eastern France (Fritz et al. 2005a; Vamberger et al. 2015), as reflected by the dominance of mitochondrial haplotypes of *E. o. galloitalica* in the eastern populations (Table 1). This introgression pattern is particularly noticeable in the Chauzon population, where the mtDNA of all turtles

corresponded to *E. o. orbicularis*, whereas using nuclear microsatellite loci the turtles were identified as admixed with *E. o. galloitalica*. This strong discrepancy can only be explained by the original presence of females of the first subspecies that bred with males of the second subspecies.

The lack of Hardy–Weinberg equilibrium detected at several microsatellite loci, as well as high F_{IS} values (significant in Pont-de-Gau and Tour-du-Valat) in the contact zone (several southern and eastern populations), reflect either current regular introgression with continuous gene flow between the two subspecies or selective mating preferring the same subspecies. Moreover, the presence of many individuals with mismatch between their microsatellite assignment and mitochondrial DNA (Table 1) suggests that recent and old (more than 5–10 generations) admixture occurred, mainly in southern populations from the Camargue (recent: 31%; old: 35%) and to a lesser extent, in eastern populations from the Provence (recent: 8%; old 27%). In contrast, for the Ardèche population (Chauzon) only old introgression was reported (recent: 0%; old: 100%). For the western populations, only recent introgression was detected (recent: 33%; old: 3%): indeed, the Rhône river system (with river arms, ponds and marshlands) and coastal regions might have acted as natural dynamic historical corridors to connect subspecies on either side of the river system and more generally, among the whole southern French contact zone in the past and still today. Even if the exchange of individuals through biological corridors between all these populations appears nowadays to be more limited due to the embankments along the Rhône river, the observed genetic structure might reflect the ancient gene flow.

In contrast to the various degrees of admixture between *E. o. galloitalica* and *E. o. orbicularis* revealed for southern and eastern populations, central and western populations are less introgressed by *E. o. galloitalica*. This subspecies occurs only in a considerable distance from central and western populations, and without any connecting waterway. Yet, although western and central populations are genetically less admixed than southern populations, they display a similar level of genetic diversity to southern and eastern populations (see allelic richness values in Table 1). This observed high diversity of western and central populations might result from much large population sizes than in the south and east, with an estimate of 50,000 pond turtles in Brenne, central France (Servan 2000) and comparable numbers in the west.

Similar to most species in Western Europe, the populations of French pond turtles might have been directly or indirectly impacted by recent and old human activities: the alteration of natural aquatic ecosystems by the drainage of marshlands and ponds, the canalisation of river systems, growing settlements and towns, and the translocation of turtles over centuries have to be mentioned here as prominent examples (Ferri and di Cerbo 2000; Bringsøe et al. 2001; Fritz et al. 2005b). Moreover, this species has also been collected for human consumption or used to prepare medical preparations or remedies (Cheylan 1998). The occurrence of old introgressed pond turtles with genetic signatures of the non-native subspecies *E. o. hellenica* in two southern populations (Aigues-Mortes and Pont-de-Gau) is probably the result of human activity (Vamberger et al. 2015). Unfortunately, the two cases described in this study are not the only populations that have been affected by old or recent introduction of alien pond turtles; other populations in southern France also contain translocated *E. o. hellenica* (Ficheux 2013; Vamberger et al. 2015). As European pond turtles can evidently reproduce over 60 years (Rollinat 1934), massive anthropogenic disturbance may last for fewer than ten generations. Indeed, as the European pond turtle is a species with a long generation time (Fritz 2001), its population genetic structure might not yet be affected by strong and recent habitat alterations (within fewer than ten generations).

The European Pond turtle is subject to numerous conservation programmes throughout Europe, including reintroduction in several cases (Fritz and Chiari 2013; Mignet et al. 2014; Canessa et al. 2016). To maintain the natural genetic differentiation throughout Europe that evolved over thousands of years, reintroduction programmes should only use the native local subspecies or individuals that resemble the genetic composition of native populations close to the reintroduction site. In this context, the precise knowledge on the genetic structure and breeding behaviour in natural populations and contact zones are essential to select genetically suitable turtles and predict the behaviour of reintroduced populations.

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CHAPTER 2

Hybridization between pond turtles subspecies in a human-mediated contact zone

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Abstract

Hybridization is a natural process mediated by environmental changes and habitat modifications and can be altered by human activities. Hybridization can promote evolutionary divergence between taxa by reinforcement of the pre-zygotic barriers to avoid the production of unfit hybrids or can prevent evolutionary divergence by the fusion of interacting lineages, the production of a novel hybrid species or the extinction of one parental species. Outcomes of hybridization are often difficult to predict: they may be species specific and context dependent and may be impacted by both genetic and local ecological factors. We investigated hybridization in a human-mediated contact zone between 3 *Emys orbicularis* subspecies and found that 83% consisted of hybrids, mainly corresponding to *E. o. hellenica* subspecies. We then compared several fitness components in hybrid and non-hybrid individuals and found no significant differences in survival rate or growth. Interestingly, individuals with the best survival rate belonged to the locally native subspecies *E. o. orbicularis*, indicating that this subspecies is highly adapted to the Swiss natural conditions. We finally provided some guidelines to manage populations with hybrids and for national conservation of the species.

Introduction

Human alteration of natural habitats disturbs and affects the integrity of geographic and reproductive barriers. Consequently, Human activities intentionally or accidentally mix formerly allopatric species and taxa and thus alter natural patterns of genetic exchange, leading to unexpected hybridization and introgression events (Allendorf et al. 2001; Crispo et al. 2011). In the absence of reproductive isolation mechanisms like pre- and post-zygotic barriers, hybridization between lineages may lead to the introgression of species and taxa (Abbott et al. 2013). Species living in sympatry may develop reproductive isolation and reinforcement mechanisms to select against maladaptive matings between lineages and against hybrid offspring (Edmands 2002; Servedio and Noor 2003). When Human activities bring together formerly allopatric taxa without reproductive isolation mechanisms, natural selection patterns against hybrids may become altered (Grant and Grant 1996). In extreme cases, a high production of hybrids may lead to the genetic swamping of one or more parental taxa (Rhymer and Simberloff 1996). When introgression affects mate recognition and when selective environment is modified, post-zygotic isolating barriers may in turn decrease patterns of natural selection against hybrids, leading to subsequent introgression events (Hendry et al. 2006).

Fitness of hybridizing populations is often difficult to predict, as hybridization may result in the formation of either beneficial or detrimental gene interactions influenced by endogenous and exogenous selection (Edmands 2002; Barton 2001): depending on genetic divergences, reproductive compatibility and relative fitness of each parental lineage, hybrid offspring may exhibit a decrease or an increase in fitness when compared to that of both parental lineages (Hwang et al. 2012). Hybrids may exhibit an increased fitness when introgression leads to new combinations of advantageous alleles, favourable epistatic interactions, overdominance (i.e. heterozygote advantage) or when it transfers adaptations from one taxon into another (Rhode and Cruzan 2005). Heterosis is often observed in the F1 generation (Lynch 1991). On the contrary, hybrids may exhibit a reduced fitness due to sterility (Palopoli and Wu 1994; Perez and Wu 1995), inappropriate breeding behaviours (Greig 1979), unsuitable habitats (Hatfield and Schluter 1999), epistasis (Edmands and Burton 1999; Burke and Arnold 2001), the loss of local adaptations (Cooke and Philipp 2005) and outbreeding depression. Outbreeding depression is generally starting from F2 and backcrosses generations, when parental gene combinations are broken up by recombination, creating deleterious interactions (Edmands et al. 2009).

Hybrid offspring may fill novel environments and niches that were previously not occupied by the native parental genotypes (Lewontin and Birch 1966; Moore 1977), especially when hybridization results in intermediate or transgressive phenotypes (Burke and Arnold 2001; Veen et al. 2001; Dittrich-Reed and Fitzpatrick 2013), like exceptional size and shape, thermal tolerance, new breeding behaviours, new resistance to infectious agents or new tolerance to biotic and abiotic factors (Burke and Arnold 2001; Veen et al. 2001; Dittrich-Reed and Fitzpatrick 2013). As the fitness of hybrids highly depends on the relative fitness of parental taxa, parental ecotypes adapted to certain environmental conditions may produce hybrid offspring adapted to the same environmental conditions. When parental taxa are intentionally or accidentally translocated into a new site, then parental taxa will be submitted to new environmental conditions. When one parental taxon is not adapted to the new local conditions, then hybrid offspring may exhibit a lower fitness and lower survival.

Introducing individuals is often proposed to rebuild populations of locally extinct taxa or to rescue small populations from inbreeding or from genetic erosion (Tallmon et al. 2004; Carlson et al. 2014). However, the introduction of individuals with low fitness or low survival in the new local conditions may produce intrinsically unfit or misadapted offspring (Storfer 1999; Allendorf et al. 2001; Huff et al. 2011). Furthermore, hybridization and introgression

are of particular concern when rare and wild taxa hybridize with their domesticated relatives selected to perform in human-mediated environments (Rhymer and Simberloff 1996; Barilani et al. 2005).

In Central Europe, as geographic barriers between taxa and lineages have been altered by Humans activities, a lot of secondary contact zones between taxa have created. Nowadays, previously allopatric taxa may hybridize and introgress, leading to hybrid offspring with better or lower fitness. In Switzerland, a human-mediated mixed population with three pond turtles (*Emys orbicularis*) subspecies may lead to introgressive hybridization. As this species benefits from conservation and reintroduction programmes in Europe, the introduction of potentially misadapted subspecies may lead to misadapted introgressive hybridization and may impact negatively the survival of the species in Switzerland.

Our aims were i) to determine if hybridization between pond turtles subspecies occur in a human-mediated mixed population, ii) to investigate survival and growth rate of native and non-native subspecies, iii) to investigate survival and growth rate of hybrid offspring, iv) to evaluate the impact of introgressive hybridization between subspecies within the framework of the national conservation programme of the species in Switzerland.

Materials and methods

Native Swiss *Emys orbicularis* populations are considered to be extinct due large consumption by Humans (Schneeweiss 1997) and to the deterioration and loss of its habitat and of nesting sites (Hotz and Broggi 1982; Fritz 2003; Kovàcs et al. 2004). However, casual observations are regularly reported in several Swiss regions, like in the region of Geneva (Meyer et al. 2009). There, pond turtles have been introduced in Moulin-de-Vert since at least 1950 (G Dändliker, pers. comm.) with 25 *E. o. hellenica* individuals from the Pô Plain (Italy) and 20 *E. o. galloitalica* individuals from Southern France. Additionally, an unknown number of *E. o. orbicularis* turtles have been introduced since the 1950s. *E. o. galloitalica* and *E. o. orbicularis* subspecies meet in a natural contact zone in southern France and hybridization is frequent (Raemy et al. 2017). The three subspecies are morphologically, genetically and behaviourally different from each other: both *E. o. hellenica* and *E. o. galloitalica* subspecies are smaller, harbour lighter colours than *E. o. orbicularis*, and breed 2 times per year with smaller clutch sizes than *E. o. orbicularis*. These elements are considered as adaptations of *E. o. hellenica* and *E. o. galloitalica* subspecies to warm Mediterranean climate. On the contrary, bigger size, darker colours and big clutch size once a year during the summer are considered

as adaptations of *E. o. orbicularis* to colder climate as they optimize warm gain through sun energy and warm temperatures allowing the eggs to hatch (Fritz 2003). Between 2002 and 2005 and between 2008 and 2012, 209 wild turtles were sampled in Moulin-de-Vert with conical fishing baskets placed perpendicularly to the banks (Cadi 2003) in 4 ponds. Morphometric parameters (length and width of the shell plate), weight, sex, age information, and sites of capture were reported for each individual. Simultaneously DNA was sampled from blood or buccal swabs (Poschadel and Möller 2004).

Total genomic DNA was extracted from blood samples and buccal swabs after an initial incubation at 56°C in ATL lysis buffer (Qiagen, Hombrechtikon, Switzerland) for 4 and 12 h respectively. DNA extraction followed the DNeasy Blood and Tissue Handbook manufacturer's handbook. PCR reactions were performed in 25-µL volumes with 2 µL DNA template, 8 µL sterile dH_2O, 12.5 µL MasterMix (MasterMix Kit, Qiagen) and 1.3 µL of each primer in an Eppendorf thermocycler (Eppendorf, Wesseling-Berzdorf, Germany). The cytochrome *b* gene (cyt *b*) was amplified using the primers mt-A and H15909 (Lenk and Wink 1997; Lenk et al. 1999). The cyt *b* sequences were analysed by Macrogen (Amsterdam, The Netherlands) on an ABI 3730XL sequencer (LifeTechnologies, Switzerland) and compared to reference sequences from GenBank (Accession numbers AJ131407-AJ131426; AM269887-AM269893; AY652865-AY652889; GU645999-GU646001) to assign them to a previously identified haploclade following Lenk et al. (1999), Fritz et al. (2005b, 2007) and Velo-Antón et al. (2011). The seven microsatellite loci, msEo2, msEo29, msEo41 (Pedall et al. 2009) and GmuB08, GmuD51, GmuD87 and GmuD114 (King and Julian 2004) were amplified following the conditions mentioned in the respective publications, with forward primers labelled with fluorescent dye. Microsatellite PCR products were analysed on an ABI 3130 sequencer (LifeTechnologies, Luzern, Switzerland) and allele sizes were scored using the program PEAKSCANNER 1.0 (LifeTechnologies, Luzern, Switzerland).

Hardy–Weinberg disequilibrium, observed (Ho) and expected (He) heterozygosities were calculated with GENALEX 6.5.1 (Peakall and Smouse 2012) and tested with Chi squared tests. Putative hybrids were detected using STRUCTURE 2.3.4 (Pritchard et al. 2000) using the admixture model with the correlated alleles setting. The likelihood values for $K = 1–5$ were estimated for 10 runs each, with 1.5×10^6 MCMC repetitions using the first 0.5×10^6 as burn-in. The most probable number of clusters was then assessed using the Evanno ΔK method (Evanno et al. 2005). Turtles with a probability of assignment $P_{assign} \geq 90\%$ were

considered as ‘pure‘, whereas individuals with P_{assign} < 90% were considered as recently introgressed (Vähä and Primmer 2006).

In order to evaluate the impact of the genetic status on the survival rate, we used Cormack-Jolly-Seber Model (CJS) using MARK 6.1 (White and Burnham 1999) and logit as link function to evaluate first if sex had an impact on the survival rate or the capturability. Secondly, haplotypes or hybridization status (see above) were implemented separately in the model to evaluate their impact on the demographic elements in the population of the natural reserve of Moulin-de-Vert.

To determine if hybridizing status, subspecies and sex have an impact on individuals' growth, a linear mixed effects analysis was performed used R (R Core Team 2013) and the package lme4 (Bates et al. 2014). Length of the shell plate was used as the exploratory variable, as it is correlated to body weight. Indeed body weight was not used as an exploratory variable because it may vary depending on whether individuals emptied (or not) their swim bladder and if females already laid their eggs (or not). Hybridizing statute (based on nDNA), subspecies (based on mtDNA haplotype) and sex (without interaction term) were used as fixed effects and intercepts for individual's number on the field as random effects. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. P-values were obtained by likelihood ratio tests of the full model with effects in question against the model without the effect in question.

Individuals were classified in generation classes F0 ("pure" individuals with a $P_{assign} \geq 90\%$), F1 (hybrid individuals with a $40\% \leq P_{assign} \leq 60\%$) and Backcrosses (hybrid individuals with a $60\% < P_{assign} < 90\%$). In order to determine if generation classes F0, F1 and Backcrosses had an impact on individual's growth rate, we performed the same linear mixed effects analysis adding the variable generation class into fixed effects.

Results

Numbers of analysed hybrid and non-hybrid turtles per subspecies are shown in Table 1. Observed and expected heterozygosities for each locus did not differ significantly ($p = 0.92$). The population possessed 6 of the 7 loci that were not in Hardy–Weinberg equilibrium ($p < 0.001$), what reflects a contact zone with current admixture or no random mating. As this population is not at the Hardy–Weinberg equilibrium, the occurrence of null alleles could not be investigated using e.g. MICROCHECKER. However, previous studies (Pedall et al. 2009;

Raemy et al. 2017) did not reveal the occurrence of null allele for the used loci. Averaged observed (0.748) and expected (0.838) heterozygosities did not differ significantly.

Mitochondrial analyses revealed that 13 turtles (6%) contained haplotypes of the subspecies *E. o. orbicularis* (haploclade II), 17 turtles (8%) haplotypes of the subspecies *E. o. galloitalica* (haploclade V) and 179 turtles (86%) haplotypes of the subspecies *E. o. hellenica* (haploclade IV; Table 1). Turtles with haplotypes of the subspecies *E. o. hellenica* were significantly more abundant than turtles with haplotypes of the subspecies *E. o. orbicularis* (χ^2 = 153; p < 0.001) *or E. o. galloitalica* ((χ^2 = 146; p < 0.001).

Table 1

Mitochondrial haplotypes and proportion of introgression following the STRUCTURE analyses of nuclear genes.

Subspecies	N individuals	N ind. assigned to clusters 1 and 2 (prob. of assignment > 90%)	% ind. assigned to clusters 1 or 2	N putative hybrids (probability of assign. < 90%)	% ind. assigned to putative hybrids
orbicularis II	13	5	2.4%	8	3.8%
hellenica	179	28	14.0%	151	73.0%
galloitalica	17	2	1.0%	15	7.2%
Total	209	35	16.7%	174	83.3%

The ΔK method suggested two as the most likely number of clusters using microsatellite loci. Only 17% of the samples represented pure individuals: five individuals (2% of the population) contained haplotypes of the subspecies *E. o. orbicularis*, 28 turtles (14%) haplotypes of the subspecies *E. o. hellenica* and 2 turtles (1%) haplotypes of *E. o. galloitalica*. On the contrary, 83% of the population was considered as hybrids between both clusters: 8 individuals contained haplotypes of the subspecies *E. o. orbicularis,* 151 haplotypes of the subspecies *E. o. hellenica* and 15 turtles haplotypes of *E. o. galloitalica*. Hybrids were significantly more abundant than non-hybrid individuals (χ^2 = 91; p < 0.001). Interestingly, proportion of hybrids was significantly higher in haplotypes belonging to the subspecies *E. o. hellenica* than in haplotypes belonging to the subspecies *E. o. orbicularis* (χ^2 = 135; p < 0.001) and *E. o. galloitalica* (χ^2 = 122; p < 0.001). Additionally, 73% of turtles with haplotypes belonging to the subspecies *E. o. hellenica* were considered as hybrids, while only 7% of *E. o. galloitalica* were considered as hybrids, even if both subspecies were released in the 1950s with a similar number of individuals.

The best model detected the impact of haplotype on survival rate [best model: Phi(mtDNA*t)p(t); AICc Weight=0.939], where the survival rate for *E. o. orbicularis* mtDNA was comprised between 0.999 and 1.000 (average = 1.000), between 0.691 and 1.000 (0.923) for *E. o. hellenica* mtDNA and between 0.474 and 1.000 (0.891) for E. *o. galloitalica* mtDNA. With the information related to the hybrid status, the best model only included the year of capture [Phi(t)p(t); AICc Weight=0.555], whereas the second-best model included the nuclear status [Phi(hybrid*t)p(t); AICc Weight=0.334], in which hybrids have slightly higher survival rates (hybrid= 0.940; non hybrid = 0.934). The best model including the F0, F1 and Backcrosses was similar [Phi(t)p(t); AICc Weight = 0.829]. Using sex as covariable, the best CJS model was for Phi(t)p(sex*t) (AICc Weight = 0.678), consequently with a survival rate related to the year (varying between 0.765 and 1.000; average=0.940) and capture probability as a function of the sex and the year (between 0.363 and 0.646; average=0.450 for the males and 0.525 and 0.793; average= 0.636 for the females). The second-best model (AICc Weight = 0.260) is very similar, just without the impact of the year on the capturability.

Linear mixed effects analysis demonstrated that hybridizing status had no significant impact on growth rate (t-value = 0.906; p = 0.368). Furthermore, growth rates were not significantly different between mtDNA haplotype (p = 0.591): growth rate of *E. o. galloitalica* was not different from *E. o. hellenica* (t-value = -1.047, p = 0.297) or from *E. o. orbicularis* (t-value = 0.150; p = 0.881). Moreover, generations classes (F0, F1 and Backcrosses) had no impact on growth rate: F0 growth rate were not different from F1 growth rate (t-value = 0.858, p = 0.391) or from Backcrosses growth rate (t-value = 0.117, p = 0.907). Sex was the only variable having an impact on individual's growth, as females were larger than males, even if the difference is only marginally significant (p = 0.068).

Discussion

Experimental work often focuses on the consequences of hybridization on early-generation crosses, as experimental work is extensive to study the impact of hybridization on long generation-time taxa (Hwang et al. 2012). Our study is then one of the first to investigate hybridization consequences on long generation-time taxa in a human-mediated population created more than 60 years ago.

The allochthonous presence of *E. o. hellenica* and *E. o. galloitalica* in the studied area reflects past introduction events in the region of Geneva. The recent human-mediated mixture

between three *Emys orbicularis* subspecies is reflected by the strong absence of Hardy-Weinberg equilibrium in the population. Indeed, some captured individuals might still belong to the individuals released in the 1950s, and thus, assumption of random mating is not fulfilled. Low number of individuals with haplotypes belonging to the *E. o. orbicularis* subspecies may be due to the low number of individuals released in this population. Unfortunately, this value remains unknown.

Interestingly, the three subspecies have a similar growth rate but not a similar survival rate: the subspecies with the best survival rate was *E. o. orbicularis*, which is actually the native subspecies northern of the Alps. Even if it is present in low number, its high survival rate indicates that *E. o. orbicularis* is well adapted to the local conditions. Allochthonous subspecies like *E. o. hellenica* and *E. o. galloitalica* seem to be differently adapted to the local conditions: the very high proportion of haplotypes belonging to *E. o. hellenica* and the high survival rate of this subspecies may indicate that this subspecies is also highly adapted to the natural conditions observed in Geneva. Indeed, released *E. o. hellenica* individuals came from the Po Plain, where temperatures and habitats are similar to the studied area (marshlands with a lot of vegetation). On the contrary, lower amounts of *E. o. galloitalica* individuals and lower survival rate within this subspecies may indicate that this subspecies is not be adapted to the ecosystems conditions in Geneva: indeed, *E. o. galloitalica* is known to inhabit mountain streams in Mediterranean ecosystems with warmer climate than in Switzerland. Consequently, our results confirm that some haplotypes are more or less adapted to specific climatic conditions and thus would have an impact in reintroduced populations.

Our study reported that hybridization events between the 3 subspecies have produced large amounts of viable and fertile hybrid offspring. Indeed, 83% of the population consist of hybrid offspring. The proportion of hybrids was higher in haplotypes belonging to the subspecies *E. o. hellenica* than in haplotypes of both other subspecies. The very high proportion of *E. o. hellenica* hybrids may reflect the natural history of *E. o. orbicularis* and *E. o. galloitalica* and some lack of reproductive isolation between the Italian subspecies and the two sympatrically living French subspecies. Naturally sympatric taxa like *E. o. orbicularis* and *E. o. galloitalica* may have developed reinforcement mechanisms and/or postzygotic barriers to avoid or limit hybridization. On the contrary, *E. o. hellenica* may not have developed barriers to reproduction to avoid hybridization with allopatric taxa. As a consequence, *E. o. hellenica* may hybridize easily with other subspecies, leading to the genetic swamping of the other subspecies.

First generation hybrids often display a higher fitness due to heterosis, whereas lower fitness in the subsequent generations is frequently observed, leading to outbreeding depression. Outbreeding is known to have deleterious effects when taxa are highly different from each other or when one taxon has a lower fitness than the other (Edmands 1999). When highly divergent populations with different fitness levels introgress, a genetic swamping may arise and low-fitness hybrids may be eradicated on the long-term. As the *E. o. galloitalica* subspecies has a lower survival rate and does not live in the same environmental conditions than the other subspecies, it seems that selection has swamped individuals with haplotypes belonging to *E. o. galloitalica* out. However, the best survival rate model reported no difference in survival between hybrids and non-hybrids, while the second-best model indicated that hybrids have a slightly higher survival rate. Additionally, our results indicate that hybrids have not a higher growth rate than non-hybrids and the different generation classes have not different growth rates. As a consequence, our results suggest that hybrids do not exhibit heterosis or outbreeding. However, our study only investigated the growth and the survival rates and not the direct fecundity.

However, long-term consequences of introgressive hybridization are generally difficult to forecast and depend on levels of genetic divergence between taxa, reproductive incompatibilities and relative fitness of the parental populations in the environment. Furthermore, outcomes of introgressive hybridization may evolve differently depending on taxa involved in the hybridizing event (Hwang et al. 2012).

Conservation biology has long considered hybrids to hold a low conservation value, particularly when hybrid offspring threaten rare native species and locally adapted populations, when hybrids suffer from outbreeding depression or when they result from human activities (Allendorf et al. 2001; Wolf et al. 2001). Recently, however, conservation value of hybrids has gained recognition, as hybridization is an important evolutionary process increasing population genetic variability and promoting adaptation and speciation (Abbott et al. 2013; Dittrich-Reed and Fitzpatrick 2013; Fitzpatrick et al. 2015).

In the future, it will be interesting to investigate hybrid genotypes rather than statistical comparisons of performances across generation classes, especially to reveal the genetic basis of increased/decreased hybrid fitness. Hybrid zone dynamics may be more complicated than previously thought, in that reinforcement and sexual selection in sympatric populations may not always reflect the complexity of all possible interactions in hybrid zones (Veen et al.

2001): in such tension zones, where a balance oscillates between hybridization and selection against hybridization, learned and plastic mate towards homospecific preferences may also occur (Veen et al. 2001). Such mate preferences should also be investigated to continue studying dynamics of contact zones.

Conclusion

Hybridization is a natural process mediated by environmental changes and habitat modifications and can be altered by human activities It can promote evolutionary divergence between taxa by reinforcement of the pre-zygotic barriers to avoid the production of unfit hybrids. On the contrary, it can prevent evolutionary divergence by the fusion of interacting lineages, the production of a novel hybrid species or the extinction of one parental species.

Our study demonstrated that hybridization occurs widely between three pond turtle's subspecies in a human-mediated contact zone. The subspecies with the higher survival rate is the native *E. o. orbicularis* subspecies, while the subspecies with the lowest survival rate is the allochthonous *E. o. galloitalica* subspecies originating from southern France. Our results may reflect some kind of reinforcement mechanisms and/or postzygotic barriers to avoid or limit hybridization between *E. o. orbicularis* and *E. o. galloitalica* but an absence of such reproductive isolation mechanisms in *E. o. hellenica*. Our results suggested that hybrids do not exhibit heterosis or outbreeding in the European pond turtle and that this species is able to swamp out misadapted individuals and to recover in new environmental conditions.

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CHAPTER 3

Detection of the European pond turtle (*Emys orbicularis*) by environmental DNA: Is eDNA adequate for reptiles?

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Abstract

Recent studies have demonstrated the potential of combining molecular technologies with environmental sampling to detect various vertebrate species in aquatic ecosystems. The European pond turtle (*Emys orbicularis*) is a threatened and elusive aquatic reptile with shy behaviour. We aimed to develop and evaluate a methodology to detect the presence of this secretive aquatic reptile in ponds from environmental water samples. First, we determined that reptilian DNA can be isolated and amplified from water samples in artificial and natural ponds with known turtle densities. Then we compared the potential of two water sampling methods (through filtration or precipitation) and found no significant differences between these approaches. Finally, we demonstrated that the eDNA concentration detected is not correlated with the number of *E. orbicularis* individuals or biomass. Detection of eDNA was higher in artificial ponds with small volumes of water or in the shallow waters of natural ponds. The eDNA-based methodology aims to detect the presence of vertebrate species, even at low densities, with better accuracy than visual observation. However, our study indicates that this method of population monitoring should be applied with caution to aquatic reptiles.

Introduction

Effective management of endangered native species and recently introduced invasive species requires efficient detection methods, especially for populations at low densities. Population estimates in freshwater habitats and marine ecosystems have recently been estimated from environmental DNA (eDNA). This approach is a rapid, cost-effective tool for applied conservation biology and could be used as an early-warning system to detect the presence of endangered species (Wilcox et al. 2013; Sigsgaard et al. 2015) and invasive species (Ficetola et al. 2008; Jerde et al. 2011; Goldberg et al. 2013; Piaggio et al. 2014). This method has been used for mammals (Foote et al. 2012; Thomsen et al. 2012a), amphibians (Ficetola et al. 2008; Goldberg et al. 2011; Thomsen et al. 2012a), invertebrates (Tréguier et al. 2014), fish (Jerde et al. 2011; Takahara et al. 2012; Kelly et al. 2014a) and reptiles (Piaggio et al. 2014).

Persistence of DNA in the environment has been intensely researched over the last decade (e.g. Willerslev and Cooper, 2005). It has been shown to persist for days or weeks in aquatic environments (Dejean et al. 2011; Thomsen et al. 2012b; Piaggio et al. 2014; Pilliod et al. 2014), and it may persist in soil for centuries or millennia (Willerslev et al. 2003; Haile et al. 2007; Yoccoz et al. 2012). Low persistence in aquatic freshwater and marine ecosystems may be explained by fragmentation (e.g. Deagle, Eveson and Jarman, 2006; Willerslev and

Cooper, 2005; Taberlet et al. 2012). Biochemical processes and DNases as well as UV-B light, temperature, salinity or pH (Lindahl, 1993; Barnes et al. 2014; Strickler, Fremier and Goldberg, 2015) can all cause fragmentation, which leads to difficulties with PCR amplification. As eDNA degrades rapidly in freshwater ecosystems, positive detection of a species indicates the recent presence of that species, which makes eDNA an important tool for species detection (Dejean et al. 2011; Foote et al. 2012; Thomsen et al. 2012a, 2015).

Recent surveys have successfully established quantitative relationships between the biomass of fish species and the relative abundance of their eDNA detected in the environment (Takahara et al. 2012; Thomsen et al. 2012a). They reveal that eDNA-based methodologies are reliable for high secreting taxa, but little is known about its applicability to lower secreting taxa like reptiles. Reliable quantification of biomass or the number of individuals may be estimated by quantifying the initial eDNA concentration from positive replicates using quantitative PCR (Ellison et al. 2006; Bustin et al. 2009). However, eDNA concentration does not always reflect the real biomass or species composition in an ecosystem (Kelly et al. 2014a). Problematic issues, such as low-quality and low-quantity DNA as well as contamination and inhibition from co-extracted substances may lead to false positive and false negative results (John, 1992; Matheson et al. 2010; McKee, Spear and Pierson, 2015). Consequently, such biases may lead to over- or under-estimates of species occurrence and thus impact conservation efforts (Kelly et al. 2014b; Thomsen and Willerslev, 2015).

Environmental DNA comes from excreted cells or tissues and as free extracellular DNA from secretions, hairs and epidermal tissues (Beja-Pereira et al. 2009 and references therein). Despite the fact that amphibians and fish are reliably detected with eDNA, little is known about the applicability of this technique to detect the presence of reptiles in aquatic ecosystems (Kelly et al. 2014a). The European pond turtle (*Emys orbicularis*) is a threatened aquatic reptile, whose distribution area has decreased dramatically during the last few centuries due to the loss of aquatic habitat and nesting sites (Fritz, 2003), to consumption by humans (Schneeweiss, 1997) and, locally, to competition from exotic species (Cadi and Joly, 2004). Current conservation and reintroduction programmes benefit to this species in several European countries (Fritz and Chiari, 2013). Due to the cryptic behaviour of this species, traditional monitoring techniques with boats and nets are time-consuming, difficult to set up, prone to false-negative detection rates (Jerde et al. 2011), and could have a strong impact on ecosystems by disturbing local fauna and flora (Biber, 2011; Tyre et al. 2003). In some cases, visual observation can be an alternative approach to determine the presence of turtles in

ponds, but this approach may lead to a large underestimation of the distribution of this species. Consequently, detecting the presence of this cryptic species by developing alternative approaches should improve the efficiency of monitoring programmes for this high priority conservation species and for aquatic reptiles in general.

We evaluated the potential of molecular techniques to detect the presence of an aquatic reptile species in environmental water samples from artificial and natural ponds, and we compared two different water sample collection methods (filtration and precipitation). In addition, we attempted to determine if eDNA concentration reflects the relative abundance or biomass of pond turtles to determine whether eDNA-based methodologies may be accurate to detect and evaluate the density of an aquatic reptile species.

Materials and Methods

We first attempted to validate our ability to detect pond turtle eDNA in water samples collected from artificial ponds and then applied this methodology to natural ponds during the summer 2015.

Sampling and extraction from captive sites

To collect eDNA from artificial ponds, 90 mL of water from six outdoor ponds in Switzerland with a known number and biomass of captive *E. orbicularis* (Table 1) was filtered through a Millipore® Sterivex™ 0.22 µm filter (Sigma-Aldrich, St. Louis, USA). Filters were then immediately stored at -80°C for further processing. To recover cellular remains and DNA from the filters, membranes were cut into small pieces and transferred into Eppendorf tubes for extraction with the QIAamp Tissue Extraction kit (Qiagen, Hombrechtikon, Switzerland) as per the manufacturer's standard protocol.

Sampling and extraction from natural sites

To collect eDNA from natural ponds, 90 mL of water from 15 sites in four different natural ponds with known turtle densities (Table 1) was filtered following the procedure described above. In order to compare the efficiency of sampling between filtration and precipitation methods, additional 90 mL water was collected simultaneously from the same locations and was uniformly distributed in six Falcon tubes each with 33.5 mL ethanol and 1.5 mL sodium acetate (Ficetola et al. 2008). Samples were then immediately stored at -80°C until further processing. To recover DNA and cellular remains from water samples, tubes were centrifuged

at 13,200 × g for 30 min at 6°C and the supernatant was discarded (Ficetola et al. 2008). Then 360 µL ATL buffer and 40 µL Proteinase K (Qiagen, Hombrechtikon, Switzerland) were used to resuspend the pellet in the first tube. This solution was then used to resuspend the pellet from the second tube. This procedure was repeated for all tubes from the same site until pellets from all the six tubes were dissolved and gathered together in the 6th tube as described by Ficetola et al. (2008). To extract DNA from the cellular remains, the solution was incubated for 3 h at 56°C. Finally, 400 µL buffer AL (Qiagen) and 400 µL ethanol were added to samples prior to elution. Further extraction steps were performed following the manufacturer's standard protocol (QIAamp Tissue Extraction; Qiagen).

Specificity of primers

Specifically designed primers eDNA_1f (5'-CCAAATATCCTTCTGAGGTGC-3') and eDNA_1r (5'-GCGTTATCTACTGAGAATCC-3') were used to amplify a 115 bp fragment of the mitochondrial *cyt b* gene. The alignment search tool BLAST (Basic Local Alignment Search Tool; Genbank, www.ncbi.nlm.nih.gov/blast) showed that these primers do not match with high scores to any published European species sequence on GenBank (www.ncbi.nlm.nih.gov/blast) except for *E. orbicularis*. Pond turtle DNA from one positive control was quantified with a Nanodrop 1000 Spectrophotometer (ThermoScientific, Wilmington, USA) and diluted from 5×10^{-1} to 5×10^{-15} µg/µL to obtain positive control dilutions. Primer specificity was tested by PCR amplification of the positive control *E. orbicularis* DNA dilutions and water samples containing pond turtle DNA. PCR was performed with 55 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s. Positive PCR products were sent to Macrogen Europe for sequencing and *cyt b* sequences were compared to reference sequences on GenBank.

Amplification by quantitative PCR

To improve the sensitivity of eDNA amplification, quantitative PCR (qPCR) using SYBR Green PCR Master Mix (Roche, Basel, Switzerland) was carried out with an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) including an initial 10 min denaturation step at 95°C, 55 cycles of denaturation at 95°C for 30 s and annealing at 56°C for 30 s on both water samples and on positive controls to set the detection threshold. Amplification of water samples as well as positive and negative controls was repeated 6 times following the multi-tube approach to account for stochasticity in low quantity or quality DNA amplification (Taberlet et al. 1996). Amplification in any of the six replicates was considered

to be positive detection when the initial DNA concentration was above the detection threshold and when sigmoidal amplification curves and melting temperatures were identical to that of positive controls. Crossing point (Cp) values of all positive replicates were determined with ABI Prism 7000 SDS software 1.1 (Applied Biosystems).

All DNA procedures were conducted using precautions required for ancient DNA and for analysing degraded DNA at low concentrations (Taberlet et al. 1996; Willerslev and Cooper, 2005; Knapp and Hofreiter, 2010). For instance, the extraction and amplification steps were performed in laboratories where no genetic work on turtles had previously been conducted. Moreover, to detect putative contamination of samples with exogenous DNA during the study, negative controls (field, DNA extraction and PCR blanks) were processed throughout the whole procedure.

Statistical analyses

In captive ponds, we tested if the detected DNA concentration was correlated with the known number of individuals and the biomass of turtles using a Spearman correlation test in R 3.3.0 (R Core team, 2016) using the average of all qPCR quantifications for each sample. In natural ponds, we tested if the detected DNA concentration was correlated with the number of turtles present at that site by the same statistical method. The number of individuals was previously estimated by traditional monitoring techniques with nets. As natural ponds have unknown depths and volumes, we did not investigate any correlation between biomass and the detected DNA concentration in natural ponds. Additionally, we visually searched for *E. orbicularis* for 5 min at each location point before sampling the water. Finally, the difference between sampling methods (filtration on membranes vs. precipitation in tubes) was analysed with a Wilcoxon rank sum test conducted with R.

Results

All PCR products from positive controls and from water samples reported only *E. orbicularis* sequences, confirming that the primers developed for our study were highly specific to this species. We succeeded in amplifying turtle DNA from water samples collected in tubes or on filters with a detection limit of 5×10^{-11} µg/µL.

In captive ponds with high turtle biomass, the eDNA-based methodology identified the presence of turtles in all five ponds with detection probabilities (the proportion of positive

PCRs per water sample) ranging from 83.3% to 100%, whereas visual observations identified the presence of turtles in only one pond. The concentration of DNA detected in water samples ranged from 5.39×10^{-8} to 2.97×10^{-2} µg/µL (Table 1).

Since we found no difference in the efficiency of the filtration method when compared to the precipitation method (V = 8; p = 0.353), we combined the data collected by both methods for further analyses.

Table 1

Rates of detection, visual observations and eDNA concentrations of *Emys orbicularis* in captive and natural ponds with filtration and precipitation water sampling methods.

Ponds	Site	N turtles / day	Visual obs.	DNA concentration µg/µL		Positive detection		Known biomass turtles g/l
				Filters	Tubes	Filters	Tubes	
Captive	Gempen 1	5	no	1.10E-05	-	5/6	-	0.1
	Gempen 2	7	yes	1.28E-02	-	6/6	-	4.375
	Gempen 3	6	no	1.59E-03	-	6/6	-	2
	Menziken 1	6	no	7.74E-03	-	6/6	-	1.5
	Menziken 2	4	no	1.04E-02	-	6/6	-	1
Natural	Pond 1	2.33	yes	2.97E-05	1.06E-06	3/6	6/6	-
	Île 1	1.67	no	-	-	0/6	0/6	-
	Île 2	6.67	no	-	-	0/6	0/6	-
	Est 1	1.33	no	1.26E-06	7.52E-07	3/6	6/6	-
	Est 2	7.00	no	-	-	0/6	0/6	-
	Est 3	6.33	no	-	-	0/6	0/6	-
	Est 4	6.67	no	4.62E-07	7.83E-07	3/6	4/6	-
	Est 5	1.00	no	-	-	0/6	0/6	-
	Est 6	12.33	no	2.74E-07	2.24E-07	4/6	3/6	-
	Hainard 1	1.33	no	-	-	0/6	0/6	-
	Hainard 2	7.33	no	-	-	0/6	0/6	-
	Hainard 3	0.67	no	6.64E-07	2.08E-04	4/6	5/6	-
	Hainard 4	5.67	no	-	4.84E-05	0/6	3/6	-
	Hainard 5	4.33	no	-	9.87E-04	0/6	5/6	-
	Hainard 6	0.33	no	-	-	0/6	0/6	-

In natural ponds, the eDNA-based methodology identified the presence of turtles with detection probabilities ranging from 25–100% (based on both sampling methods) in seven of the 15 natural sites, whereas visual observations identified the presence of turtles at only one site. Mean DNA concentrations ranged from 1.68×10^{-7} to 9.87×10^{-4} µg /µL. No turtle DNA was detected in the other eight sites, even though all of them are known to be inhabited by *E. orbicularis* (Table 1). No correlation was found between the detected DNA concentration in captive ponds and number of turtles (r = 0.198; p = 0.749) or the turtle biomass (r = 0.641; p = 0.244). Similarly, we did not find a significant correlation between the number of turtles and the DNA concentration in natural ponds (r = -0.031; p = 0.912). Interestingly, sites where turtle DNA was detected were shallow waters with vegetation. On the contrary, samples from sites with a 2–4 m depth provided low detection probabilities (0–25%), despite the fact that they are sites known to be inhabited by turtles.

Discussion

We successfully detected the presence of a reptile with an eDNA-based methodology in aquatic ecosystems. In captive ponds, turtles were found with high detection probabilities. In natural ponds, turtles were detected at half of the sites, even though all of them are known to be occupied by a high density population. Interestingly, turtle DNA was only detected in shallow waters with aquatic vegetation. Detecting the presence of turtles in these areas may reflect the microhabitat used by the species: shallow waters are warmer and richer in vegetation than deeper waters, and these habitats are known to be used by turtles for feeding and refuging (Fritz, 2003). At the 15 natural ponds sites, we found no correlation between the estimated number of turtles per site and the DNA concentration. This contradicts recent surveys establishing quantitative relationships between species biomass and relative abundance of detected eDNA in the environment (Takahara et al. 2012; Thomsen et al. 2012a; Pilliod et al. 2014). However, this may again reflect the microhabitat used by this species: pond turtles are easily captured with nets in deep water as they move along the banks, but they do not use deep waters for feeding, basking or refuging. They use shallow waters for their main activities and deep waters just to move between optimal shallow waters. Additionally, turtles may be more detectable in shallow waters as faeces from aquatic macrofauna spend a limited time period suspended in the water column (Turner et al. 2014). DNA from faeces would be more detectable in shallow waters or deeper in the water column.

Visual observation revealed the presence of turtles in only one site while traditional capture surveys found turtles in all 15 sites. As our eDNA-based methodology detected turtles in only half of the natural sites, our results contain a high proportion of false negative results. False negatives are frequent in environmental studies and can occur when eDNA is degraded and at low concentration (Darling and Mahon, 2011; Ficetola et al. 2015), or when water samples contain high quantities of environmental particles (Volkmann et al. 2007). Statistical methodologies have been developed, however, to counterbalance the impact of false negatives in studies where no precise information about the presence of a species in a population is available (Schmidt et al. 2013). Based on our results, we suggest that aquatic turtle monitoring based on eDNA may be more reliable than surveys based on direct observation, but may be less efficient than traditional net-based surveys. In contrast with several other studies (Ficetola et al. 2008; Thomsen et al. 2012a), lower rates of detection by eDNA methods were also found for aquatic mammals (Foote et al. 2012).

Aquatic reptiles are likely to be more difficult to detect with an eDNA-based methodology than other higher secreting aquatic taxa such as amphibians and fish, which can be detected even at low densities (Ficetola et al. 2008; Jerde et al. 2011; Thomsen et al. 2012b). Lower detection probabilities for turtles may be due to the presence of scutes rather than epithelial cells and, consequently, to their low DNA shedding rates (see discussion in Kelly et al. 2014a). Moreover, biomass and surface area are known to be influential factors, as a group of small individuals sheds more genetic material than a single large individual (Foote et al. 2012; Thomsen et al. 2012b; Kelly et al. 2014a). Additionally, effectiveness of the eDNA method may be influenced by temporal and spatial variability of eDNA within the sampling area, non-homogenous mixing or heterogeneity in the distribution of the sample within the water column, variation in the rates of dilution and diffusion in the environment, and variables affecting DNA degradation such as temperature, pH or UV-B light (Goldberg et al. 2011; Mahon et al. 2013; Bohmann et al. 2014; Strickler, Fremier and Goldberg, 2015). Finally, surveys establishing quantitative relationships between species biomass and relative abundance of detected eDNA (Takahara et al. 2012; Thomsen et al. 2012a; Pilliod et al. 2014) have been applied to taxa other than reptiles and to a reptile species under controlled conditions. Thus, our results may suggest that environmental studies on reptiles in natural conditions may be less suitable for detection by eDNA methods than other higher secreting taxa.

To our knowledge, our study is the first to compare two different water sampling methods (filtration and precipitation) for eDNA collection from natural ponds. We found no significant differences in their efficiency, which is contradictory to Turner et al. (2014). Accumulation of particulate matter in pond water, such as sediments, humic substances and vegetation remains, may decrease the filter membrane efficiency when collecting eDNA. However, the large numbers of particles in pond water readily precipitate during the precipitation method and may provide a useful source of eDNA. Consequently, filters may be more appropriate for stream waters (that generally contain a very limited amount of particles) as they allow eDNA to be collected from larger volumes of water more easily than the precipitation method. Therefore, we recommend using the precipitation method for ponds and the filtration method for stream waters.

Conclusion

Genetic markers are valuable tools for monitoring biodiversity, detecting and identifying species, and can provide valuable information for the management and conservation of species and ecosystems (Schwartz, Luikart and Waples, 2007; Foote et al. 2012). However, species detection in eDNA-based surveys and traditional monitoring surveys are likely to be imperfect, which can lead to an underestimation of species distribution (Schmidt et al. 2013) and poor management decisions. Surveys based on eDNA require careful attention due to biases in detection. Differential DNA shedding rates and/or preferential amplification of species and the use of microhabitats need to be considered when planning environmental studies and interpreting eDNA results. Despite these potential drawbacks, eDNA-based monitoring surveys are predicted to have a promising role in the future of environmental management (Kelly et al. 2014b). However, this promising tool may be less suitable for low secreting taxa, such as reptiles, than for higher secreting taxa. As a consequence, we argue that eDNA methods should be used to complement, rather than replace, traditional monitoring approaches for aquatic reptiles.

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CHAPTER 4

Conservation activities for European pond turtles (*Emys orbicularis*) in Switzerland

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Conservation activities for European pond turtles (*Emys orbicularis*) in Switzerland

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Introduction

During the Holocene expansion, Switzerland was probably recolonized by *Emys orbicularis orbicularis* haplotype IIa north of the Alps and by *E. orbicularis hellenica* haplotype IVa (as defined in Lenk et al., 1999) south of the Alps (Fritz, 2003). Due to the climatic conditions in Switzerland, the suitable habitats to species occurrence are limited to the Swiss Plateau (north of the Alps) and the Ticino region (south of the Alps) at an elevation below 500m. Historically, the species was probably common in numerous aquatic habitats such as lakes, ponds, marshlands and rivers as suggested by subfossil records. However, human activity (e.g. fishing) and the destruction of aquatic habitats and nesting sites have led to the decline of *E. orbicularis* populations in Switzerland.

Native populations may have survived in Switzerland until the 17th or 18th centuries (Fatio, 1872; Fritz, 2003). Observations conducted during the 20th century indicate that the individuals currently living in Switzerland are likely to be escaped or released from captivity and not representing relict populations (Monney and Meyer, 2008). This assumption is corroborated by genetic analyses. Presently, only one breeding population of about 350 allochthonous individuals is reported from the canton of Geneva. This population results from successive introductions of turtles of haploclades II, IV and V during the 1950's. The possibility that autochthonous individuals survived in this location is unlikely (Raemy, 2010). Due to the low number of individuals and to the occurrence of a single breeding population in Switzerland, *E. orbicularis* is considered as critically endangered (Monney and Meyer, 2005) and as a target species for national conservation programs.

Conservation projects

Location: Swiss Plateau (below 500m of elevation) and Ticino

Project duration: 2010 - present

Fundings: Federal Office for the Environment, Geneva Canton (Direction générale de la nature et du paysage), Communauté d'Intérêts pour les Tortues en Suisse (CITS/SIGS)

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Local Emys conservation problems: In Switzerland, due to the high human density on the Swiss Plateau and in the Ticino region, the natural suitable habitats (aquatic ecosystems) and nesting sites of *Emys orbicularis* have seriously been altered and only a few locations may be currently still suitable to the survivorship of this species. Furthermore, available aquatic habitats often lack basking and nesting sites. Therefore, an ecosystems restoration is needed to ensure the presence of suitable habitats for this species. Furthermore, various allochthonous subspecies are currently found in Switzerland, probably due to imported individuals of different subspecies or haploclades that were either released or escaped into the wild.

Conservation project activities and main results: The goals of the *Emys* conservation project in Switzerland are 1) to recreate stable and viable populations in favourable sites 2) to manage and protect favourable sites with regards to the requirements of this species (management and creation of nesting sites) 3) to promote scientific research on the species to improve the chances of successful reintroductions 4) to improve the genetic purity of the species by reintroducing native subspecies (*E. o. orbicularis* IIa north of the Alps and *E. o. hellenica* IVa south of the Alps). Among favourable locations selected by preliminary habitat analyses, the site of Prés Bordon (about 10 km of Geneva) was chosen for the first reintroduction programme in Switzerland. This natural reserve (at an altitude of 480m) comprises three interconnected ponds restored in 2008 with aquatic and riparian vegetation, basking and nesting sites. Presence of surrounding natural forest and wetlands would allow further colonisation in other ponds and wetlands. In 2010, 14 juveniles previously genotyped and belonging to the native subspecies were released in the natural reserve of Prés Bordon. Schools of the surrounding villages, medias and politicians actively participated to the release in order to increase awareness to the protection of this species and to the preservation of aquatic ecosystems. Juveniles were obtained from the Association Protection et Récupération des Tortues (PRT) and from two private breeders of Swiss *Emys*. All individuals were marked with specific notches in the carapace and equipped with transmitters to evaluate their settlement and survival during one year. Preliminary results suggest that all individuals survived and settled in the site. A monitoring using nets is planned every year to estimate survival and growth rates. Further studies will be conducted to evaluate breeding success and the location of nesting sites..

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GENERAL DISCUSSION

Hybridization between distinct subspecies was investigated by combining nuclear and mitochondrial markers to evaluate if distinct pond turtles subspecies hybridized in a natural contact zone and to what extent introgression occured between different subspecies living sympatrically (**Chapter 1**). Mitochondrial results agreed with previous studies about the phylogeography of the European pond turtle and confirmed the occurrence of a contact zone between *E. o. orbicularis* and *E. o. galloitalica* subspecies for southeastern France (Lenk et al. 1999; Fritz et al. 2005, 2007; Pedall et al. 2011). More precisely, I reported a more complex pattern of introgression between the two subspecies than previously thought: in the southeastern contact zone, the combination of mtDNA and nuclear markers allowed to attest eastwards directed nuclear introgression from *E. o. orbicularis* into *E. o. galloitalica*. These results underlined that variable nuclear markers are highly informative to investigate contact zones. Moreover, mismatches between nDNA and mtDNA probably reflected both inheritance modes and colonization histories by this species: as males are more prone to dispersal (Ficheux 2013), southern France populations might have been first colonised by *E. o. orbicularis* during the Holocene southwards through the Rhône valley (as indicated by mtDNA) and might have been later invaded by *E. o. galloitalica* (as indicated by the nuclear introgression) expanding their range westwards. Current introgression with continuous gene flow or selective mating towards the same subspecies might be reflected by the absence of Hardy–Weinberg equilibrium for several microsatellite loci and by high F_{IS} values in the southeastern contact zone. The Rhône river system and coastal regions might have acted together as natural dynamic historical corridors among the whole southern French contact zone. However, as turtles are slow evolving taxa (Avise et al. 1992), genetic consequences of embankments along the Rhône River and settlements may not be reflected in the genetic structure and diversity of pond turtles yet. Interestingly, even if western and central populations were not located in contact zones between different lineages, they displayed a similar level of genetic diversity to populations from contact zones. This high genetic diversity of western and central populations might be due to large populations (Servan 2000).

In the second part of my thesis, I investigated hybridization levels and hybridization outcomes between pond turtle subspecies in a human-mediated contact zone in the region of Geneva (**Chapter 2**): I reported the presence of the locally native *E. o. orbicularis* turtle subspecies and the presence of two locally allochthonous *E. o. hellenica* and *E. o. galloitalica* turtle

subspecies. I found that the population consisted of more than 83% hybrids and was strongly not at the Hardy-Weinberg equilibrium, reflecting the recent human-mediated mixture. Surprisingly, the number of individuals with haplotypes belonging to the subspecies *E. o. hellenica* was higher than the number of individuals with haplotypes belonging to the other two subspecies, even if *E. o. hellenica* and *E. o. galloitalica* were released with approximately the same amount of individuals in the 1950s. The higher proportion of *E. o. hellenica* hybrids might reflect some lack of reproductive isolation between the Italian subspecies and the two other *E. o. orbicularis* and *E. o. galloitalica* subspecies. Indeed, the latter two subspecies might have developed some kind of reproductive isolation mechanisms to avoid high rates of misadapted hybrids. Interestingly, the subspecies with the best survival rate (1.000) was *E. o. orbicularis*, attesting that the native *E. o. orbicularis* subspecies is highly adapted to the environmental conditions on the Swiss Plateau. Furthermore, results reported a high survival rate (0.923) for *E. o. hellenica* too, corroborating that this subspecies is adapted to the Swiss natural conditions too, as it is native to Tessin. The high survival rates of *E. o. orbicularis* and *E. o. hellenica* subspecies may be due to their life history: their ancestors were released in the 1950s from regions with similar habitats than those from the region of Geneva. On the contrary, lower amounts of *E. o. galloitalica* individuals and lower survival rate (0.891) suggested that this subspecies might not be adapted to the Swiss environmental conditions: indeed, *E. o. galloitalica* inhabits mountain streams and warmer ecosystems along the Mediterranean Sea coast, with habitat characteristics and temperatures different from those in Switzerland. Therefore, I reported some outbreeding in the population of Geneva, especially for *E. o. galloitalica* and I reported that both Swiss native *E. o. orbicularis* and *E. o. hellenica* are well adapted to the Swiss environmental conditions.

In **Chapter 3**, I developed a novel genetic tool based on the eDNA methodology to pursue genetic studies on pond turtles in aquatic ecosystems. Combining water samples with RT-PCR succeeded in the detection of pond turtles from aquatic ecosystems. However, detecting turtles in artificial ponds was more efficient than in natural ponds, as turtles were only detected in half of the natural ponds, even if all of them were known to harbour turtles. Interestingly, turtle DNA was detected in shallow waters with aquatic vegetation, what may reflect the turtle microhabitat: shallow waters are warmer and richer in vegetation than deeper waters and are mainly used by turtles for feeding and refuging (Fritz 2003). The estimated number of turtles per site was not correlated to the observed DNA concentration, what contradicted surveys establishing quantitative relationships between species biomass and

relative abundance of detected eDNA in the environment (Takahara et al. 2012; Pilliod et al. 2014). I suggested that the eDNA-based methodologies were less efficient when applied to low secreting taxa like reptiles, but that they might be suitable for high secreting taxa like amphibians and fishes (Ficetola et al. 2008; Jerde et al. 2011). Lower detection probabilities for turtles might be due to the presence of scutes rather than epithelial cells and, consequently, to lower DNA shedding rates (Kelly et al. 2014). Finally, I found no significant differences in the efficiency of both precipitation and filtration methods, what contradicted Turner et al. (2014) who had found that the precipitation method was more efficient in rivers: indeed, the accumulation of particulate matter from ponds to the filter membrane might decrease the efficiency of the filtration method. Therefore, I recommend using the precipitation method when sampling water from ponds and using the filtration method for stream waters.

Outlook

This thesis revealed that *E. orbicularis* subspecies hybridize in natural and human-mediated contact zones. However, hybridization patterns between natural contact zones may differ from those in human-mediated contact zones. Subspecies that evolved sympatrically with other subspecies might have developed some kind of reproductive isolation mechanisms, while subspecies that did not evolve sympatrically with other subspecies in contact zones might not have developed such mechanisms. This is particularly sensitive for the human-mediated mixed population in the region of Geneva, where hybrid individuals are reported more frequently than in the natural contact zone in Southern France. As *Emys orbicularis* subspecies harbour different behaviours and haploclades (Fritz 2003) and may have developed some selection against hybrids (this thesis), some speciation processes may arise between *E. o. orbicularis* and *E. o. galloitalica* subspecies on the long-term.

Outcomes of hybridization and patterns of introgression may evolve differently depending on genetic divergences between taxa or populations and on their relative fitness (Hwang et al. 2012). As turtles are long-living organisms, it remains difficult to predict introgression impacts over many generations. However, this thesis indicated that individuals with haplotypes belonging to the Swiss native *E. o. orbicularis and E. o. hellenica* subspecies are well adapted to the Swiss environmental conditions. However, as hybrid fitness is driven by the relative levels of outbreeding and levels of heterosis, and is impacted by interactions with environmental conditions, hybridization outcomes may be different if other subspecies are

involved in a hybridizing event under other environmental conditions. Developing genetic methods to investigate hybrid genotypes rather than statistical comparisons of performances across generation classes will bring interesting insights to determine the genetic basis of increased/decreased fitness in hybrids.

Environmental DNA-based monitoring surveys are predicted to have a promising role for environmental management in the future, especially for the detection of rare or invasive species (Kelly et al. 2014). Moreover, if eDNA-based approaches are combined with next-generation sequencing methodologies, then captivating horizons may open to population genetics surveys: combining universal primers with eDNA samples will provide more detailed and valuable information on the proportion of lineages in a population and on the genetic structure of this population. However, species detection based on traditional monitoring surveys and on eDNA methodologies are likely to be imperfect. This underestimation of the species distribution may lead to poor management decisions, especially for low secreting taxa like reptiles. As a consequence, eDNA-based methodologies should be used to supplement, rather than supplant, traditional monitoring approaches for aquatic reptiles. In order to counteract the underestimation of the species distribution when working with eDNA-based methodologies or traditional monitoring techniques, applying site occupancy models may help to deal with imperfect detection during field work and laboratory procedures (Schmidt et al. 2013).

To maintain the natural genetic differentiation between populations that evolved since the Holocene recolonization of Europe and to maintain dynamics of contact zones, conservation programmes should always consider the genetic composition of native populations. In natural contact zones where introgressive hybridization is reported, hybrid individuals should also be reintroduced to mirror the hybrid composition of local populations. In this context, the genetic structure, levels of divergence between taxa, breeding behaviours, levels of reproductive incompatibilities and the relative fitness of the parental populations in the environment are of primary consideration for selecting genetically suitable turtles. In Switzerland, only the *E. o. orbicularis* subspecies should be reintroduced by the national conservation programme for the Swiss Plateau to reflect the natural genetic pattern of *E. o. orbicularis* and its high survival rate. Moreover, as *E. o. hellenica* subspecies is native in Tessin and had high survival rate too, this subspecies should be released in Tessin, corresponding to its natural distribution range. However, as *E. o. galloitalica* subspecies is not native to Switzerland and had lower survival rate than the other subspecies under the Swiss conditions, care should be taken when this

subspecies is reported in a site where a reintroduction event is planned. As the *E. o. hellenica* subspecies may lack some reproductive isolation mechanisms, care should also be taken when the presence of this subspecies is reported, to avoid introgressive hybridization between genetically and behaviourally different pond turtle subspecies.

As Human-mediated hybridisation is increasing worldwide (Allendorf et al. 2001), determining guidelines for hybrid management is essential (Jackiw et al. 2015) and identifying adequate taxonomic and genetic delimitation of conservation units will be important challenges to improve conservation efforts (Frankham et al. 2009). Traditionally, hybrids have long been considered to hold a low conservation value (Allendorf et al. 2001), while they have recently gained recognition for species conservation (Fitzpatrick and Schaffer 2007; Fitzpatrick et al. 2015): as hybrids might harbour novel environmental adaptations and show novel ecological and evolutionary responses to environmental stressors (Edmands 2007; Hamilton and Miller 2015; Hoffmann et al. 2015), they may provide functional novelty and species resilience to environmental changes.

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SUPPLEMENTARY MATERIALS

TO CHAPTER 1

ELECTRONIC SUPPLEMENTARY MATERIAL

Hybridisation between turtle subspecies: a case study with the European Pond Turtle.

Conservation Genetics

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Figure S1: Likelihoods under the different settings for K.
Figure S2: STRUCTURE results**.**
Table S1: FST values for each population.
Table S2: Parameters and results of the simulations conducted with MIGRATE 3.6.8 (Beerli and Palczewski, 2010) with the 6 populations from the southern contact zone Pont-de-Gau (PdG), Aigues-Mortes (AM), Marais des Baux (MdB), Bolmon (Bo), Tour-du-Valat (TdV) and Vigueirat (Vig).

Figure S1: Likelihoods under the different settings for K

Likelihoods under the different settings for K given by STRUCTURE.

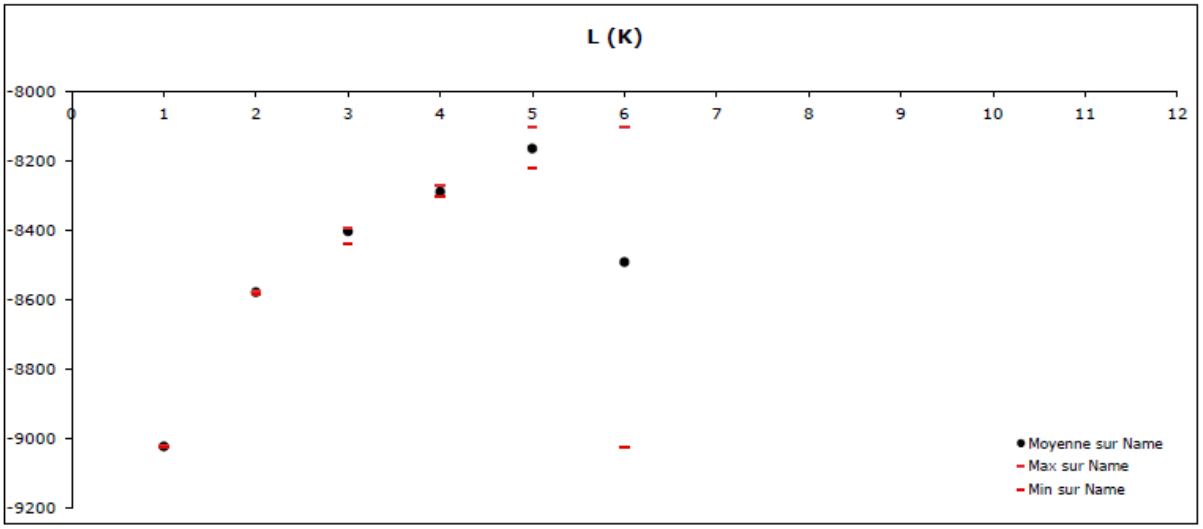
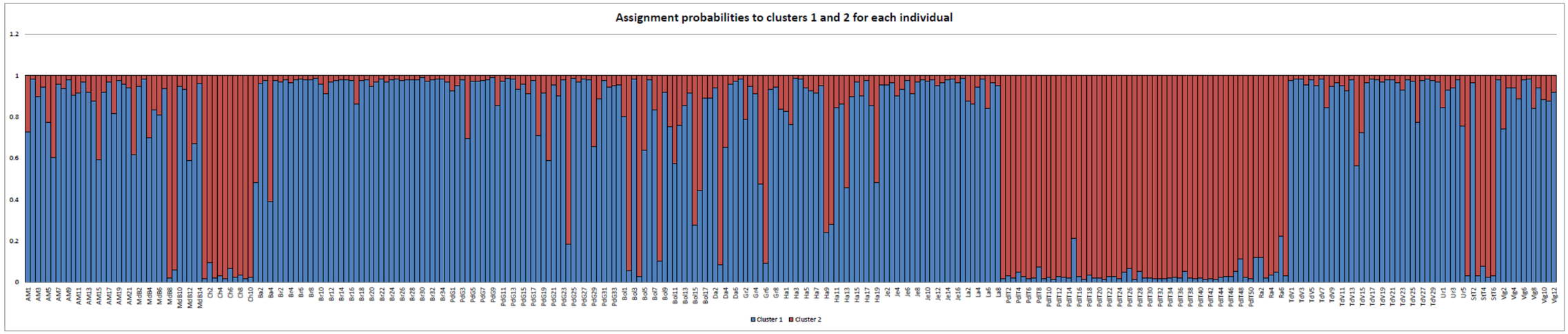


Figure S2: STRUCTURE results

Assignment of individuals to the first cluster (blue) or to the second cluster (red). Turtles with a probability of assignment (P_{assign}) > 90% were considered as pure (non-introgressed), whereas individuals with P_{assign} < 90% were considered as introgressed. Individuals are listed by population under the figure.



Individuals	Populations
AM1-AM21	Aigues-Mortes
Ba1-Ba4	Bassussarry
Bol1-Bol17	Bolmon
Br1-Br35	Brenne
Ch1-Ch10	Chauzon
Da1-Da6	Dax
Gr1-Gr9	Graveyron
Ha1-Ha19	Haillan
Je1-Je16	Jemaye
La1-La8	Labenne
MdB1-MdB14	Marais des Baux
PdG1-PdG34	Pont-de-Gau
PdlT1-PdlT50	Plan-de-la-Tour
Ra1-Ra7	Ramatuelle
StT1-StT6	St-Tropez
TdV1-TdV30	Tour-du-Valat
Ur1-Ur5	Urt
Vig1-Vig12	Vigueirat

Table S1: FST values for each population

	Jemay	Haillan	Graveyron	Dax	Labenne	Bassussarry	Urt	Aigues-Mortes	Pont-de-Gau	Tour-du-Valat	Vigueirat	Bolmon	Marais des Baux	Plan-de-la-Tour	Ramatuelle	St-Tropez	Chauzon
Brenne	0.1521**	0.0942**	0.0889**	0.1032*	0.0643**	0.1042 NS	0.0793**	0.074 **	0.1106 **	0.1038 **	0.0771 **	0.1234 **	0.1512 **	0.1508 **	0.1536 **	0.1195 **	0.2732 **
Jemay		0.1038**	0.1124**	0.1563 NS	0.0586 *	0.1641 NS	0.1105 NS	0.1485 **	0.1084 **	0.1147 **	0.1336 **	0.1193 **	0.1384 **	0.196 **	0.1971 **	0.1696 NS	0.3582 NS
Haillan			0.0117 NS	0.0179 NS	0.0207 NS	0.0496 NS	0.0176 NS	0.0508 **	0.0471 **	0.0565 **	0.0115 NS	0.0447 **	0.0316 **	0.0787 **	0.0691 **	0.0402 NS	0.2684 *
Graveyron				0.0014 NS	0.0265 NS	0.0633 NS	0.0151 NS	0.0369 **	0.0422 **	0.0511 **	0.0143 NS	0.0687 **	0.0463 *	0.0555 **	0.0428 NS	0.0452 NS	0.2892 NS
Dax					0.0372 NS	0.0477 NS	0.02 NS	0.0485 NS	0.0422 NS	0.0407 NS	0.0113 NS	0.0785 NS	0.0814 NS	0.0664 NS	0.0495 NS	0.0665 NS	0.2925 NS
Labenne						0.0749 NS	-0.0001 NS	0.039 *	0.0356 **	0.0438 **	0.0297 NS	0.038 NS	0.0578 NS	0.1054 **	0.0998 NS	0.0561 NS	0.2846 NS
Bassussarry							0.0557 NS	0.0772 NS	0.0794 NS	0.0608 NS	0.0318 NS	0.1331 NS	0.1238 NS	0.1479 NS	0.1312 NS	0.1058 NS	0.3393 NS
Urt								0.0592 **	0.0537 **	0.0472 *	0.0173 NS	0.0524 *	0.0405 NS	0.0729 **	0.0605 NS	0.0595 NS	0.2783 NS
Aigues-Mortes									0.0698 **	0.0654 **	0.0309 **	0.0943 **	0.0846 **	0.1047 **	0.0878 **	0.0631 NS	0.2545 *
Pont-de-Gau										0.0288 **	0.0585 **	0.0574 **	0.0738 **	0.1267 **	0.0966 **	0.1067 **	0.2589 **
Tour-du-Valat											0.0387 **	0.0812 **	0.0839 **	0.1184 **	0.0932 **	0.0843 **	0.2636 *
Vigueirat												0.0749 **	0.0541 *	0.0594 **	0.0468 *	0.0323 NS	0.2545 NS
Bolmon													0.0753 **	0.1039 **	0.1081 **	0.0971 NS	0.2999 NS
Marais des Baux														0.1029 **	0.1113 *	0.1072 NS	0.2899 NS
Plan-de-la-Tour															0.0115 NS	0.018 NS	0.2645 *
Ramatuelle																-0.0048 NS	0.3132 NS
St-Tropez																	0.2855 NS

Table S2: **Migrate results**.

Parameters and results of the simulations using MIGRATE 3.6.8 (Beerli and Palczewski 2010) with the six populations from the contact zone Pont-de-Gau (PdG), Aigues-Mortes (AM), Marais des Baux (MdB), Bolmon (Bo), Tour-du-Valat (TdV) and Vigueirat (Vig).

Parameter	2.50%	25.00%	Mode	75.00%	97.50%	Median	Mean
Θ_{PdG}	0.045	0.047	0.048	0.049	0.052	0.049	0.051
Θ_{AM}	0.045	0.048	0.049	0.050	0.053	0.049	0.050
Θ_{Al}	0.052	0.065	0.066	0.067	0.069	0.062	0.059
Θ_{ChlM}	0.007	0.009	0.014	0.015	0.017	0.015	0.025
Θ_{TdV}	0.067	0.075	0.077	0.079	0.082	0.072	0.067
Θ_{Vig}	0.019	0.026	0.027	0.030	0.035	0.030	0.035
$M_{AM->PdG}$	0.000	14.67	30.33	44.67	60.00	40.33	189.5
$M_{Al->PdG}$	228.0	314.7	335.0	355.3	371.3	332.3	365.8
$M_{ChlM->PdG}$	0.000	8.667	21.67	34.00	58.00	29.67	149.7
$M_{TdV->PdG}$	0.000	13.33	22.33	30.67	45.33	23.67	29.49
$M_{Vig->PdG}$	304.0	334.0	353.7	373.3	419.3	355.7	317.3
$M_{PdG->AM}$	56.67	70.00	86.33	104.0	132.0	99.00	218.7
$M_{Al->AM}$	438.7	454.7	471.0	486.7	601.3	476.3	460.8
$M_{ChlM->AM}$	0.000	3.333	21.67	38.67	56.00	36.33	180.6
$M_{TdV->AM}$	10.00	28.00	47.00	66.00	76.67	146.3	209.3
$M_{Vig->AM}$	2.667	16.00	36.3	100.0	122.0	318.3	397.8
$M_{PdG->Al}$	264.0	433.3	475.7	497.3	516.7	431.0	432.4
$M_{AM->Al}$	0.000	7.333	30.33	57.33	80.67	57.00	220.7
$M_{ChlM->Al}$	417.3	432.0	440.3	448.7	462.7	441.0	436.8
$M_{TdV->Al}$	382.7	402.0	423.0	442.0	456.0	225.0	258.8
$M_{Vig->Al}$	92.67	118.0	147.0	200.0	216.7	189.7	312.6
$M_{PdG->ChlM}$	303.3	362.0	384.3	406.7	568.0	389.7	407.4
$M_{AM->ChlM}$	409.3	440.0	452.3	464.0	489.3	455.7	472.0
$M_{Al->ChlM}$	0.000	2.667	11.67	20.67	42.67	18.33	82.25
$M_{TdV->ChlM}$	0.000	43.33	56.33	68.67	90.00	56.33	94.52
$M_{Vig->ChlM}$	227.3	283.3	301.7	320.0	360.0	297.0	297.6
$M_{PdG->TdV}$	2.667	28.67	40.33	50.00	76.67	43.00	76.74
$M_{AM->TdV}$	3.333	24.67	37.67	49.33	71.33	43.67	167.5
$M_{Al->TdV}$	0.000	5.333	17.67	28.67	54.67	25.00	155.5
$M_{ChlM->TdV}$	0.000	9.333	17.67	26.00	41.33	19.67	27.83
$M_{Vig->TdV}$	0.000	8.000	16.33	24.00	38.00	18.33	30.22
$M_{PdG->Vig}$	0.000	12.67	21.00	30.00	63.33	23.67	46.67
$M_{AM->Vig}$	0.000	0.000	12.33	27.33	165.3	145.7	254.5
$M_{Al->Vig}$	461.3	500.7	514.3	526.0	547.3	511.0	469.4
$M_{ChlM->Vig}$	0.000	2.667	10.33	18.00	38.00	15.00	50.34
$M_{TdV->Vig}$	545.3	619.3	641.0	661.3	753.3	619.0	442.3

Parameters used

Markov chain settings:	Long chain
Number of chains	1
Recorded steps [a]	100
Increment (record every x step [b]	20

Number of concurrent chains (replicates) [c]	1
Visited (sampled) parameter values [a*b*c]	2000
Number of discard trees per chain (burn-in)	10000

Prior distribution for parameter

Parameter	Prior	Minimum	Mean*	Maximum	Delta	Bins
Theta	Uniform	0.000	0.010	0.100	0.010	1500
M	Uniform	0.000	100.0	1000	100.0	1500

CURRICULUM VITAE

Personalities

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Education

PhD thesis 2010-2017
Departement of Environmental Sciences, Section of Conservation Biology, University of Basel

Master in Parasitology and Ecoethology 2007-2010
University of Neuchâtel

Bachelor in Biology and Environnemental Sciences 2004-2007
University of Fribourg

Working experience

Scientific collaborator (Biodiversity, Ecological Networks, Landscape Quality) 2016-2017
Swiss Federal Office for Agriculture, Bern, 90%

Manager for the sector Environment and Biodiversity 2013-2016
Chamber for Agriculture Fribourg, Granges-Paccot, 60%

Scientific collaborator for Marie Garnier (politician FR) 2012-2013
Institute for Agriculture, Grangeneuve, 50%

Practical "Swiss Strategy for Biodiversity" 2012
Swiss Federal Office for the Environment, 80%

Practical Pro Natura 2011
Center Pro Natura, Champ-Pittet, 80%

Assistant in Biology
Department of Biology, University of Fribourg 2007-2010

Personal experiences

Council member WWF Fribourg 2006-2017
Monitor Panda Club Fribourg 2006-2017
Volunteer for the cantonal Service for Nature and Landscape Fribourg 2006-2017

During my studies I attended lectures by the University of Basel:

- Research Seminars (2010-2017)
- Literature Seminars (2010-2015)
- Assistant for block courses (2010-2017)

During my studies I gave conferences during the following congresses:

- National congress of karch (Bern, 2016)
- International congress for Sustainability (Basel, 2012)
- International congress of the European Herpetological Society (Luxemburg, 2011)
- International congress of the French Herpetological Society (Grenoble, 2010)
- International congress for the European pond turtle (Châteauroux, 2010)
- National congress of karch (Schwyz, 2010)