RESEARCH ARTICLE

Chelonian challenge: three alien species from North America are moving their reproductive boundaries in Central Europe

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Citation: Tietz B, Penner J, Vamberger M (2023) Chelonian challenge: three alien species from North America are moving their reproductive boundaries in Central Europe. NeoBiota 82: 1–21. https://doi.org/10.3897/neobiota.82.87264

Abstract

Biological invasions by alien species have substantial economic impacts and are a major driver of the ongoing decline and loss of biodiversity. Through humans, the North American pond slider (*Trachemys scripta*) has acquired a global distribution over the last decades and is currently listed among the worst invasive reptile species. However, in more recent times, other freshwater chelonian species have increasingly been recorded far outside their native distribution ranges as well, not only on the same continent but also on others. Despite that, the impact of alien chelonians on their respective new ecosystems remains unclear. The long-term effects and severity of impacts of alien populations mostly depend on whether they ultimately succeed in establishing themselves. This is not entirely resolved for chelonians in Central Europe. To answer that, we investigated wild populations of three non-native chelonian species from North America in Germany (*Pseudemys concinna*, *Graptemys pseudogeographica* and *Trachemys scripta*) applying population genetic approaches. We revealed the successful reproduction of all three species in Germany and provide the very first record for the reproduction of *P. concinna* and *G. pseudogeographica* in a temperate continental climate zone outside their native distribution. Based on our unambiguous evidence of natural reproduction, we call for dedicated studies to verify how widespread established populations are and to investigate the existing and potential impacts of all three species in a range of ecosystems along a climatic gradient. Such data is urgently needed to revise the current risk assessments of non-native chelonians, especially in Central European countries.

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Introduction

Biological invasions by alien species have substantial economic impacts (Essl et al. 2020; Diagne et al. 2021; Soto et al. 2022) and are also a major driver of the ongoing decline and loss of biodiversity (Butchart et al. 2010; Ripple et al. 2017; IPBES 2019a, b; Seebens et al. 2021). Despite that, the number of alien species is growing continuously (Pyšek et al. 2020). And regardless of an existing unifying framework for biological invasions (Blackburn et al. 2011), the terms "alien", "casual/introduced", "naturalised/established" and "invasive" are often not applied correctly in numerous scientific and non-scientific publications. This makes common language a challenge. In the following, for simplicity reasons, we consider a species alien if human actions enabled them to overcome biogeographical barriers and invasive once a population becomes established, e.g. exhibiting regular reproduction. Whether a species outside its native distributional range should be categorised as alien or invasive is our main underlying study question.

To highlight the serious impacts of invasive species, the IUCN Invasive Species Specialist Group's (ISSG) lists the "100 World's Worst Invasive Alien Species" (Lowe et al. 2000). It aims to illustrate a wide variety of examples from microorganisms, fungi, plants, invertebrates and vertebrates (Lowe et al. 2000). At first glance, it seems paradoxical that some of the taxa listed therein are also the world's most threatened ones. For example, chelonians (Reptilia: Order Testudines) are among the most imperiled vertebrates on the planet (Lovich et al. 2018; Rhodin et al. 2018; Stanford et al. 2018; Cox et al. 2022) with over 60% of the species listed as threatened by extinction (IUCN 2021; Cox et al. 2022). At the same time the pond slider *Trachemys scripta* (Thunberg in Schoepff, 1792) is listed among the worst invasive reptile species (Lowe et al. 2000). Furthermore, at least three other freshwater chelonians have a substantial risk of becoming invasive (Bugter et al. 2011).

Trachemys scripta is native to south-eastern North America (Ernst and Lovich 2009; Vamberger et al. 2020) and meanwhile has acquired a global distribution, being widespread on all continents except Antarctica (Kikillus et al. 2010; Uetz et al. 2022). Due to their popularity as pets, especially the subspecies *T. scripta elegans*, they were massively imported to Europe in the 1980s and 1990s (Arvy and Servan 1998; Ernst and Lovich 2009; Vamberger et al. 2012) and released in water bodies. In 1997, the European Union banned imports of *T. scripta elegans* (Commission Regulation EC 338/1997). Sales of individuals born in EU member states were not forbidden until 2016 (EU Regulation 1143/2014 on Invasive Alien Species), which then included all

subspecies of *T. scripta*. However, by then *T. scripta* was already widely present in water bodies all over Europe (Kikillus et al. 2010; Standfuss et al. 2016; Uetz et al. 2022 and references therein). Unfortunately, these regulations seemed to have caused a shift in demand for freshwater chelonians. Two other subspecies, *T. scripta scripta* and *T. scripta troostii*, as well as several other species, especially of the genera *Pelodiscus*, *Pseudemys*, *Graptemys*, and *Chrysemys*, have replaced *T. scripta elegans* in the pet trade (Rhodin et al. 2010; Bugter et al. 2011; Carretero and Pinya 2011; Lipovšek 2013; Brejcha et al. 2014; Escoriza et al. 2021; Uetz et al. 2022 and references therein) and are also illegally released into numerous freshwater bodies around the world.

The impacts of these alien chelonians on their respective ecosystems remain largely unclear (see also Bugter et al. 2011). So far, studies have focused on direct impacts on other chelonians, e.g. the European pond terrapin *Emys orbicularis* (Cadi and Joly 2004), which is of conservation concern in many countries in Europe. Despite the proximal causes remaining unknown, in an experimental setup native *E. orbicularis* showed weight loss and high mortality when kept together with *T. scripta*. The most likely suggestion seems that the larger alien species exclude the smaller native ones from basking spots and thus the latter suffer from suboptimal thermoregulation (Cadi and Joly 2004). There are also hints that native amphibian larvae recognise native freshwater chelonians as predators but not alien ones (Polo-Cavia et al. 2010) so alien *T. scripta* might have feeding advantages. Other effects are not studied but experimental evidence suggests a key role of chelonians in ecosystem functioning, altering, for example, sediment accumulation, leaf litter decomposition rates and abundance of invertebrates (Lindsay et al. 2013; Dupuis-Desormeaux et al. 2022). This indicates potentially severe impacts outside their native ranges. However, before studying impacts we must consider the question of whether alien populations are established, i.e. whether they are regularly reproducing in the wild outside their native range (Bugter et al. 2011).

We investigated wild populations of three non-native chelonian species (river cooters *Pseudemys concinna*, false map turtles *Graptemys pseudogeographica* and pond sliders *Trachemys scripta*) using population genetic approaches with 14 microsatellite loci and performing parentage analyses. Our assumptions are that reproduction in the wild occurs, if (i) juveniles are found in the wild, (ii) closely related individuals are recorded and (iii) that a population has established itself when at least half of the studied markers are in Hardy–Weinberg equilibrium (HWE) (following Standfuss et al. 2016). HWE is reached, when allele and genotype frequencies in a population remain constant from generation to generation, thus there is an absence of other influences on the population (e.g. immigration). However, HWE cannot be achieved if continuously new alleles are added to a population, in other words through continuous releases of non-native chelonians. In addition, detection of unrelated individuals would suggest repeated releases of chelonians and no reproduction in their exotic environments. Herein we unravel whether these three species have formed self-sustaining populations in south-western Germany outside their native distribution ranges, which would be the first time for *P. concinna* and *G. pseudogeographica* and the most northern record for *T. scripta*.

Methods

Study sites

Based on informal reports of relatively large populations of pond sliders *Trachemys scripta*, in situ inspections of several water bodies, and observations of hatchlings of *T. scripta* in Kehl (Pieh and Laufer 2006; Schradin 2020), two study sites in Germany were selected (Fig. 1; Suppl. material 1: fig. S1). Our first study site, "Flückigersee" (48°00'38"N, 7°49'06"E, abbreviated FR) is a dredging lake located in the middle of the city of Freiburg im Breisgau, categorised as a semi-natural lake with a

Figure 1. Map of Baden-Württemberg, Germany, with locations and satellite photos of both study sites. Hill shade symbolises elevation, forest cover is illustrated in green and urban areas in light red. The main map shows the location of the sites within Baden-Württemberg and top left within Germany. Map was created with QGIS (QGIS Development Team 2020).

size of 11.2 ha, and maximum depth of 26.8 m (LUBW 2020) (Fig. 1). Its elevation is 240 m a.s.l., annual mean temperature in Freiburg is 10.3 °C (Climate-Data.org 2020). The second study site "Altrhein" (Fig. 1; Suppl. material 1: fig. S1) is a 3.3 ha standing oxbow lake located in the city of Kehl (48°34'04"N, 7°48'37"E, abbreviated KE), disconnected from the current course of the river Rhine (LUBW 2020). Its elevation is 139 m a.s.l. and the city's annual mean temperature is 11.1 °C (Climate-Data.org 2020). Both water bodies are located in urban parks and are completely surrounded by residential areas.

Fieldwork

Fieldwork was conducted between May and August 2020. We caught chelonians opportunistically by hand, dip netting, a non-baited basking trap and ten baited funnel-traps with modified elastic entrances to enable large individuals to enter the traps. Funnel-traps were baited with chicken heart, chicken liver, beef or mixtures of anchovies, mackerel, codfish liver and cat food. They were placed in shallow areas, tied to nearby vegetation, using buoys to ensure that the traps could not submerge completely.

Captured living chelonians had blood drawn from the sub-carapacial space above the neck for genetic analyses. Tissue samples were taken only from dead individuals. We used Whatman FTA Cards (GE Healthcare Life Sciences, Chalfont St Giles, GB) and ethanol for preservation of blood samples. Sex was determined for individuals above 9 cm carapax length, using secondary sexual characteristics such as elongated claws on forelimbs (only present in males) and position of the cloacal opening (in females closer to the shell then in males) (Ernst and Lovich 2009). Age was estimated as a combination of the number of growth rings and shell abrasions (Govedič et al. 2020). Juveniles represent individuals of up to the age of 2 years (2 growth rings). Based on shell abrasion, adults were divided into three classes: young adults, middle-aged adults and old adults (Meeske 2006; Vamberger and Kos 2011; see Table 1). All aspects of field work were approved under permit number 35-9185.81/G-20/06 by the "Regierungspräsidium Freiburg, Abteilung 3" of the German federal state of Baden-Württemberg.

DNA extraction, PCR and microsatellites

We extracted genomic DNA from FTA cards by using the illustra Tissue and Cells genomicPrep Mini Spin Kit (GE Healthcare Life Sciences). For extraction of DNA from blood, tissue and cloaca swabs preserved in ethanol we used the innuPREP Blood DNA Mini Kit (Analytik Jena GmbH). For amplification of microsatellite DNA, three Multiplex-PCRs (MP 1–3; Suppl. material 1: table S1) were performed using the Qiagen Type-it Microsatellite PCR Kit (QIAGEN GmbH). Thermocycling conditions were as follows: one cycle of initial denaturation (95 °C; 5 min), 30 cycles of denaturation (95 °C; 30 sec), annealing (55 °C; 90 sec) and elongation (72 °C; 30 sec) and one cycle of final elongation (60 °C; 30 min). For reaction mixes we followed the protocol of Standfuss et al. (2016). Fragment length analysis was conducted on an ABI 3130xl

Table 1. Number of chelonians caught and analysed genetically, sorted by population and split by age classes and sex for all three species analysed. Sex determination of hatchlings and subadults is not possible, due to the absence of distinct sexual characters.

Population	Total	Adult females	Adult males	Sub	Ha
P. c. (FR)	33	21 (14 OA, 5 MA, 2 YA)	3 (2 OA, 0 MA, 1 YA)	3	6
G. p. (FR)	25	11 (8 OA, 3 MA, 0 YA)	5 (1 OA, 1 MA, 3 YA)	6	3
T. s. (FR)	71	35 (14 OA, 15 MA, 6 YA)	12 (5 OA, 4 MA, 3 YA)	20	4
P. c. (KE)	2	0	0	2	0
T. s. (KE)	56	21 (7 OA, 6 MA, 8 YA)	8 (2 OA, 2 MA, 4YA)	21	6

P. c. = *Pseudemys concinna*, *G. p.* = *Graptemys pseudogeographica*, *T. s.* = *Trachemys scripta*, FR = Freiburg, KE = Kehl, Sub = subadult, Ha = hatchling, OA = old adults, MA = middle-aged adults, YA = young adults; see methods for further details.

Genetic Analyzer (Life Technologies). For final determination of fragment lengths, we used the software PEAK SCANNER 1.0 (Applied Biosystems). Errors in genotyping were minimised by re-amplification of samples that showed weak or missing signals.

Cross-amplification tests of microsatellites

First we tested the applicability of the 14 microsatellite loci (Suppl. material 1: table S1) for one individual per species of *Pseudemys concinna* and *Graptemys pseudogeographica* as these were originally developed for *Trachemys scripta* (Simison et al. 2013) through cross-amplification tests. For PCR reaction we used a primer concentration of 0.025 mM (Biomers.net, Ulm, Germany) and otherwise followed the protocol of Standfuss et al. (2016).

PCRs were conducted under thermocycling conditions provided in Standfuss et al. (2016). In case of amplification, the presence of the microsatellites was confirmed by sequencing the PCR products with primers in both directions. PCR products were purified with the ExoSAP-IT enzymatic cleanup (Thermofisher, Waltham, USA) and sequenced using the reverse primer of each locus on an ABI 3130xl using the Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The applicability of each microsatellite loci on *Pseudemys concinna* and *Graptemys pseudogeographica* was approved by confirming the presence of the expected repeat motives, checked with BIOEDIT (Hall 2011).

Genetic diversity indices and cluster analysis

We used CONVERT 1.31 (Glaubitz 2004), PEAK SCANNER 1.0 (Applied Biosystems), CERVUS 3.0 (Kalinowski et al. 2007) and ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010) to analyse microsatellite data (AMOVA) and calculate genetic diversity indices for all three species. Genetic cluster analyses were performed for *T. scripta* from FR and KE using an unsupervised Bayesian clustering approach, implemented in the software STRUCTURE 2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009) to analyse whether the two populations correspond to separate clusters and form established and breeding populations. STRUCTURE searches for populations in Hardy-Weinberg

equilibrium (HWE) and linkage equilibrium. In the analyses we applied the admixture model and correlated allele frequencies and set the upper bound for calculations arbitrarily to $K = 10$. Although we sampled two populations, the upper bound was set higher to exclude potential source populations from which the animals could be released. Because MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) suggested the presence of null alleles (Suppl. material 1: table S2), data were corrected for null alleles according to Falush et al. (2007). The most likely number of populations (K) was determined by using the Ln P(D) (mean likelihood of K) values (Pritchard et al. 2000) and the ΔK method (Evanno et al. 2005), implemented in the software STRUCTURE HARVESTER (Earl and vonHoldt 2012). We repeated calculations 10 times for each K using a MCMC chain of 750 000 generations for each run, including a burn-in of 250 000 generations. Population structuring and individual admixture were visualised using the software DISTRUCT 1.1 (Rosenberg 2004). Following Randi (2008), we categorised individuals with proportions of cluster membership below 80% as having mixed ancestries.

Kinship analysis

We calculated the most likely relationships between individuals of each species at each study site. Therefore, we applied a maximum likelihood approach for pairwise estimates of relatedness and computed Wright's coefficient (r) of relatedness, implemented in ML-RELATE (Kalinowski et al. 2006). Null alleles detected by MICRO-CHECKER were accommodated by ML-RELATE. Kinship analyses were conducted for respective populations of *Pseudemys concinna*, *Graptemys pseudogeographica* and *Trachemys scripta*, from FR and one population of *Trachemys scripta* and two individuals of *Pseudemys concinna* from KE. Analyses were conducted for all relationships available in the software (U = unrelated, HS = half sibling, FS = full sibling and PO = parent-offspring). Confidence level for estimated relationships in ML-RELATE was set at 95% by running 100 000 simulations. When ML-RELATE suggested more than one relationship, we executed a specific hypothesis test for two a priori relationships by means of a likelihood ratio test. We checked all genetically identified relationships against our morphological data (e.g. age class; see Table 1) for potential errors (such as offspring older than presumed parent); no such erroneous classification was observed.

Results

In total, we sampled 33 individuals of *Pseudemys concinna*, 25 of *Graptemys pseudogeographica* and 71 of *Trachemys scripta* from FR and 56 *Trachemys scripta* and 2 *Pseudemys concinna* from KE (for more details see Table 1). Subspecies of *T. scripta* were assigned as follows: FR: 42 *T. s. elegan*s, 16 *T. s. scripta*, 9 *T. s. elegans × scripta* hybrids and 4 *T. scripta* which could not be assigned to a subspecies or hybrid; KE: 37 *T. s. elegans*, 6 *T. s. scripta*, 8 *T. s. elegans × scripta* hybrids and 5 *T. scripta* which could not be assigned to a subspecies or hybrid.

Cross-amplification tests of microsatellites

Out of 14 microsatellite loci, originally developed for *T. scripta* (Suppl. material 1: table S3), only one failed to amplify (Tsc297) in *P. concinna*, while all amplified for *G. pseudogeographica*. All analysed microsatellite loci turned out to be polymorphic for *G. pseudogeographica* (Suppl. material 1: tables S2, S3) while for *P. concinna* locus Tsc108 was monomorphic (Suppl. material 1: table S3). Accordingly, loci Tsc297 and Tsc108 were excluded from further analyses for *P. concinna*.

Genetic diversity indices

The highest average number of alleles per locus ($A_\emptyset$) was revealed in *T. scripta* FR (15.1) followed by *T. scripta* KE (13.1), *P. concinna* FR (9.1) and *G. pseudogeographica* FR (9) (Table 2). MICROCHECKER detected null alleles in all analysed species (Table 2). Numbers of private alleles ranged from a minimum of 12 in *G. pseudogeographica* (FR) to a maximum of 23 in *T. scripta* (FR). More than half of the loci were in Hardy-Weinberg equilibrium (HWE) in *P. concinna* (FR: 8/12), *G. pseudogeographica* (FR: 7/14) and *T. scripta* (FR: 9/14), while only four out of 14 were in HWE in *T. scripta* in KE (Table 2).

Genetic cluster analysis

Using all 14 microsatellite loci and the correction for null alleles in STRUCTURE, we examined whether *T. scripta* from each site (FR and KE) correspond to a population in Hardy–Weinberg and linkage equilibrium. Ln P(D) values and the ΔK method suggested $K = 2$ being the most likely number of clusters (Suppl. material 1: fig. S2), each corresponding to the respective study site (Fig. 2). However, several individuals from KE (yellow cluster; Fig. 2) clustered within corresponding individuals from FR (red cluster Fig. 2). A standard AMOVA revealed a statistically significant molecular difference between the two populations of *T. scripta* (FR and KE) of 2.01% (Fst-value: 0.02; p < 0.001).

Table 2. Genetic diversity indicators of all four chelonian populations, based on 12 microsatellite loci for *P. concinna* and 14 microsatellite loci for *G. pseudogeographica* and *T. scripta*.

Population	A_N	$A_\emptyset$	A_0	A_p	$H_{E\emptyset}$	$H_{O\emptyset}$	HWE_N	PO	FS	HS
P. concinna (FR)	109	9.1	2	20	0.70	0.75	8	7	14	48
G. pseudogeographica (FR)	126	9	3	12	0.72	0.79	7	2	18	32
T. scripta (FR)	212	15.1	4	23	0.76	0.88	9	12	18	154
T. scripta (KE)	184	13.1	5	13	0.77	0.86	4	7	49	125

FR Freiburg, ***KE*** Kehl, A_N number of alleles, $A_\emptyset$ average number of alleles, A_0 number of loci with null alleles, A_p private alleles, $H_{E\emptyset}$ average of expected heterozygosity, $H_{O\emptyset}$ average of observed heterozygosity, HWE_N number of loci in Hardy-Weinberg equilibrium, **PO** number of parent-offspring-relationships, **FS** number of full-sibling -relationships, **HS** number of half-sibling relationships.
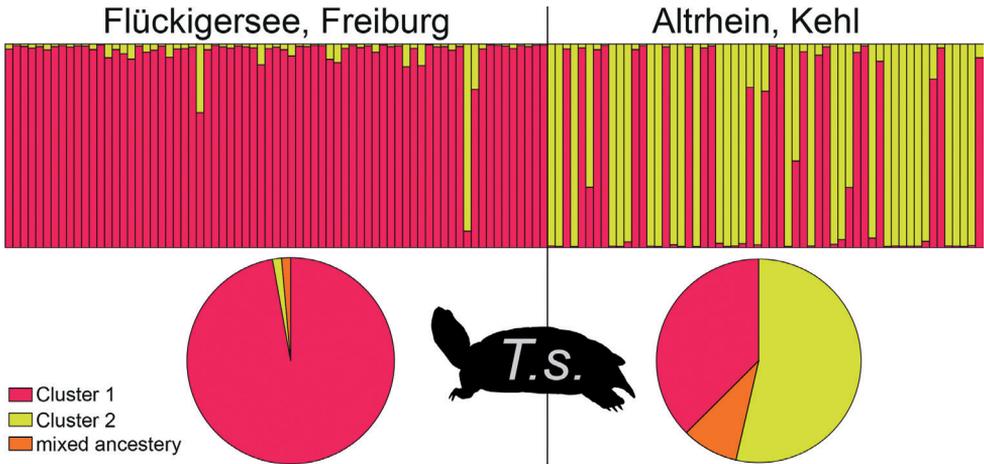
Figure 2. Population structuring in *Trachemys scripta* (*T.s.*) for *K* = 2 from the STRUCTURE run with the highest probability value. Revealed cluster (red, yellow) are presented in distinct colours. Each vertical bar represents one individual and its calculated proportion of cluster membership. Colours of pie charts correspond to STRUCTURE clusters; orange slices represent chelonians with mixed ancestry (percentages).

Kinship analysis

We detected the full variety of kinship relationships within all analysed populations. Parent-offspring-relationships were confirmed for all three species (Table 2, Suppl. material 1: tables S4–S7). Analysis of *P. concinna* from FR revealed kinship relationships among 28 of 34 (82%) sampled individuals (Suppl. material 1: table S4). The two analysed subadult individuals from KE showed a full-sibling-relationship; so far no hatchlings of *P. concinna* have been detected in KE. Kinship relationships were revealed for 18 of 25 (72%) sampled *G. pseudogeographica* (Suppl. material 1: table S5). In *T. scripta*, 58 of 71 (82%) analysed individuals from FR (Suppl. material 1: table S6) and 45 of 57 (79%) from KE (Suppl. material 1: table S7) showed kinship relations with at least one other individual. We genetically confirmed hybridisation between two subspecies of *T. scripta* in FR for one hatchling (no. 22367), detecting a parent-offspring-relationship for a male *T. s. elegans* (no. 22300) and a female *T. s. scripta* (no. 22248) (Suppl. material 1: table S6). Correspondingly, this hatchling exhibited intermediate morphological characters of both subspecies (see Suppl. material 1: fig. S3).

Discussion

For the first time we genetically confirm successful reproduction of three alien chelonian species in Germany. For two species, *Pseudemys conncina* and *Graptemys pseudogeographica*, reproduction in Germany (FR) is the first record in a temperate

continental region and for each species it is the second outside their native distribution range in North America (see below). The detected full-sibling-relationship between the two analysed subadult individuals from KE does not prove reproduction in KE, but it can be suggested. This is in line with our capture data, and so far we have not detected hatchlings of *P. concinna* in KE. Both species are popular in the European pet trade and are occasionally noted as alien species in the wild (Ottonello et al. 2014; Foglini and Salvi 2017; Ferri et al. 2020; Uetz et al. 2022 and references therein).

For *Trachemys scripta*, our genetic confirmation of suspected natural reproduction in FR and KE are the first ones for Germany, the northernmost for the species in Europe to date and the second one for a temperate continental region. Successful reproduction and self-sustaining populations of *Trachemys scripta* in Europe were previously known from Mediterranean regions (e.g. Cadi et al. 2004; Perez-Santigosa et al. 2008; Ficetola et al. 2012; Sancho and Lacomba 2016; Vecchioni et al. accepted) and the temperate continental climatic zone of Slovenia (Standfuss et al. 2016). In Germany, hatchlings of *T. scripta* have been previously sighted and documented in KE (Pieh and Laufer 2006; Schradin 2020). Egg laying (without observations of hatching events) as well as increasing numbers of juveniles were reported by Schradin (2020). Thus Pieh and Laufer (2006) and Schradin (2020) concluded that successful reproduction occurs. For *P. conncina* behavioural observations and a single juvenile in an animal shelter suggested successful reproduction under semi-natural conditions in Catalonia in Spain (Soler and Martínez-Silvestre 2020). For *G. pseudogeographica* two nesting events with partly successful hatching events in urban parks in Brescia and Milano, Italy, were observed but not published (Ferri et al. 2021).

Herewith, our data confirms these previous assumptions. Not only do we provide evidence for reproduction of three species of alien chelonians, derived from parentage analyses confirming numerous relationships (parent-offspring, full-sibling and half-sibling) between individuals (Suppl. material 1: tables S4–S7), our calculated genetic diversity indices reveal HWE for the majority of markers (Table 2; Suppl. material 1: tables S2, S3). The sampled populations seem to represent established populations, meaning they are populations with no external influences on population growth and age composition, i.e. no releases of new individuals.

For *T. scripta*, this assumption is supported by the similarity of diversity indices between both sites, especially the average heterozygosity (*T. scripta* FR: 0.88; KE: 0.86 in our study) compared to the native populations (e.g. 0.81 in Simison et al. 2013). Even though sample sizes of *P. concinna* and *G. pseudogeographica* were lower than for *T. scripta*, the numbers of loci in Hardy-Weinberg-Equilibrium are similar among all three species in FR (Table 2) and similar to populations of *T. scripta* in Slovenia (Standfuss et al. 2016). With the exception of *T. scripta* in KE, the observed high numbers of loci in HWE are remarkable, considering the anthropogenic origin of the studied populations. In general, chelonians mature sexually relatively late, especially females, between 8 to 12 years in *G. pseudogeographica* (3–4 years in males), 13 to 24 years for *P. concinna* (6 years in males) and 6 to 8 years in *T. scripta* (2–5 years in males) (Ernst and Lovich 2009). The continuous release of non-native chelonians counteracts the potential to achieve complete HWE (Standfuss et al. 2016). Nevertheless, our STRUCTURE analysis of

T. scripta populations from FR and KE revealed two most probable clusters, each corresponding to one sampling site. The population in FR seems to be already longer established than the one in KE, which is indicated by its genotypic structure (Fig. 2) and number of loci in HWE (Table 2) but no direct observational data is available.

In KE *T. scripta* was very common in the Altrhein until 2004 when the water body was restored and the population declined (Pieh and Laufer 2006). Only a single juvenile *T. scripta elegans* was found in 2004 (Pieh and Laufer 2006). During our fieldwork in 2020 we instantly detected numerous juveniles (also hatchlings) at basking sites and caught seven hatchlings and 18 sub-adults in 8 days. Evidently, the population in Kehl has increased within the last approximately 15 years, indicating that it is relatively young (see also Schradin 2020).

For successful establishment of invasive species, a viable sex ratio is important. Our data (see Table 1) indicates a sex bias towards females in adult chelonians but we believe, without having data to support our view, that males in our study system, especially in *T scripta*, are more difficult to catch than females. As males can mate with several females we do not believe that the observed sex ratio is a limiting factor and a female bias seems to be common in chelonians (Ewert and Nelson 1991; Ewert et al. 2004). Although we had no means of verifying the sex of subadult or hatchling chelonians, young adult males were caught in all three species. All three species exhibit "temperature dependent sex determination" (TSD) and therefore there could be a link between establishment at new localities and viable sex ratios. TSD is quite complex and relatively little studied across a large number of chelonian species. Changing climatic conditions, either through global change or relocation of species into novel environments, do not necessarily relate to different sex ratios in offspring. This is because molecular mechanisms, that are still being untangled, may play a significant role. A candidate gene for sex determination in *T. scripta* has previously been suggested (Ge et al. 2018). Environmental proxies influencing TSD in chelonians can be mean, range and variance of temperature at specific time periods (Girondot et al. 2010). In addition, ambient temperature is the most crucial factor but rarely known (Cornejo-Páramo et al. 2020). In contrast, there are publications showing that species with TSD can adapt to different climates, for example behaviourally through varying depths at which eggs are laid, locations of nest sites and timing of egg laying (Ewert et al. 2005; Schwanz and Janzen 2008; Refsnider and Janzen 2012; Pike 2013). Also embryos seem to have the possibility to thermoregulate inside their eggs (Shine and Du 2018). Another important point is that most models estimating pivotal temperatures, i.e. thresholds above or below only one sex is produced, still have to be validated through field data because they generally assume constant temperatures during the whole incubation period (e.g. Girondot 1999; Godfrey et al. 2003; Hulin et al. 2009) which is not necessarily always the case. Even if single clutches produce predominantly one sex, population dynamics of TSD are not well known (Ewert and Nelson 1991; Ewert et al. 2005) and it is unclear to our knowledge how that translates into viable sex ratios. There is even the suggestion that highly variable environments during development of embryos could facilitate adaptation at later life stages (Jonsson et al. 2022). So novel climates and TSD alone do not necessarily lead to sex biases and biases in viable

sex ratios. More field measurements at clutch sites across a range of climatic zones are required to reveal if, and how, viable sex ratios are achieved.

Overall, our results demonstrate the ability of three alien chelonian species to reproduce and establish viable populations in two sites in the Upper Rhine Plain in Baden-Württemberg, south-western Germany, which is considered to be one of the warmest regions in Germany. Both sites are urban habitats but alien chelonians are found in a large variety of water bodies, ranging from urban to natural, even within protected areas (Turtle Spotter 2020; pers. obs.). Therefore, it needs to be assessed whether unnoticed reproduction might have occurred also in other regions, in other alien species and more natural habitat types. For example, two hatchlings of *Chrysemys picta bellii* were observed near Speyer, Germany (Fritz and Lehmann 2002) and egg depositions of *T. scripta* were recently observed in more northern regions of Germany, e.g. Essen/Ruhr (Rautenberg and Schlüpmann 2018) and Saarbrücken (Francke pers. comm.). This is especially important in habitats where endangered species could be affected. Direct effects are potential competition with native chelonians. So far, observations indicate that *T. scripta* has competitive superiority to the endangered *E. orbicularis*, e.g. in procuring food (Nishizawa et al. 2014) and basking behaviour (Cadi and Joly 2004; Polo-Cavia et al. 2010, 2015). It was also demonstrated that *T. scripta* has physiological advantages, like faster chemosensory responses and superior thermoregulation (Polo-Cavia et al. 2008, 2009, 2012). Therefore, the abundance of alien chelonians might threaten the survival of endangered native populations of *E. orbicularis* in north-eastern Germany (Brandenburg) which harbours the last relict populations (Schneeweiß and Wolf 2009) but also in Europe in general. In addition, ongoing successful reintroduction programmes of *E. orbicularis* (Fritz and Chiari 2013) can be jeopardised by alien chelonians. In Germany, reintroduction efforts of *E. orbicularis* have been made in the federal states of Hessen and Rheinland-Pfalz (Fritz and Chiari 2013), in comparable latitudes where egg depositions of *T. scripta* were observed (Rautenberg and Schlüpmann 2018, Francke pers. comm.).

The almost omnivorous feeding behaviours of many alien chelonians might also have direct effects, indicating that rare and threatened native species of flora and fauna could by preyed upon. However, so far no data regarding food items of alien chelonians in central Europe are available. Besides these direct effects, a number of indirect effects are plausible and gaining research attention in recent times. For example, there is the risk of alien chelonians introducing and acting as reservoirs for novel diseases and parasites (Shen et al. 2011; Gong et al. 2014; Héritier et al. 2017). *T. scripta* is a known carrier of several salmonella and pathogens (Shen et al. 2011; Gong et al. 2014) and is also vulnerable to ranavirus (Moore et al. 2014). Cases of parasite transfer (e.g. helminths) from alien to native chelonians are already known from Europe (Héritier et al. 2017). Data about indirect impacts of other alien chelonians in Europe, besides *T. scripta*, are missing completely. In addition to negative effects, it was recently argued that alien chelonians in general, and *T. scripta elegans* in particular, might have the potential to offer ecosystem services in degraded ecosystems which would otherwise be lacking (Dupuis-Desormeaux et al. 2022). Thus, a differentiated view on alien chelonians in a range of habitats is called for.

Currently, the legislative restrictions for the pet trade, for example within the European Union, constrains only *T. scripta*. The other two species, *P. concinna* and *G. pseudogeographica*, are not included, and thus they are legally imported and available. It has to be assessed how widespread these species are in ecosystems outside their native range and whether control of the legal trade is necessary. Nevertheless, the focus should be laid on developing a diverse set of large public outreach campaigns to raise awareness of potential harmful impacts of releasing pets into the wild, for both the pet and the ecosystem it is released into (Teillac-Deschamps et al. 2009; Masin et al. 2014). Another step in a similar vein would be the further establishment of a "certificate of competence" (German: "Sachkundenachweis") for keeping exotic pets. During such a course potential keepers learn what resources are required to care for these animals adequately and the harm caused by illegal releases. This is already successfully established for aquarium and terrarium hobbyists in Germany (https://www.sachkunde-vda-dght.de/) and could become part of a general legal requirement. Education and public outreach can be accompanied by measures such as obligatory PIT tags (passive integrated transponder) for chelonians which can allow identification of their origin if found in the wild, thereby allowing the prosecution of illegal releases. Furthermore, a "conservation fee" could be charged when selling chelonians. Such a fee would increase costs for the keepers, hopefully lead to more careful considerations before purchasing these animals and it could be used to finance further research and fund rescue centres for released chelonians. In general, a more coordinated and positive approach to keeping exotic pets has numerous positive side effects (for example, see the recent efforts by Citizen Conservation https://citizen-conservation.org/?lang=en) such as raising awareness about the ongoing biodiversity crisis, emphasising responsible keepers and at the same time saving threatened species from extinction.

In conclusion, our results provide evidence for the novel establishment of four populations of alien chelonians belonging to three species in a temperate climate zone and thereby confirming earlier risk assessments (see Bugter et al. 2011). Remarkably, even *G. pseudogeographica* which is sensitive to cold (Ernst and Lovich 2009) reproduces in Germany and indicates that the risk assessment of *T. scripta* and other non-native chelonians species (see Bugter et al. 2011; Masin et al. 2014) should be generally revised based on scientific evidence, professional long-term monitoring efforts and if necessary adjusted accordingly, especially in Central European countries.

Acknowledgements

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Supplementary material 1

Supplementary information

Authors: Benno Tietz, Johannes Penner, Melita Vamberger

Data type: tables and figures (pdf file)

Explanation note: ID, multiplex-PCR numbers, annealing temperatures, repeat motifs, fragment sizes, fluorescent labelling of all used microsatellite loci. Genetic diversity indices of *Trachemys scripta* populations of Freiburg and Kehl. Genetic diversity indices of *Pseudemys concinna* and *Graptemys pseudogeographica*. Kinship-relationship-matrix of *Pseudemys concinna* individuals, generated by ML-RELATE. Kinship-relationship-matrix for *Graptemys pseudogeographica* individuals from Freiburg, generated by ML-RELATE. Kinship-relationship-matrix of *Trachemys scripta* individuals from Freiburg, generated by ML-RELATE. Kinship-relationship-matrix of *Trachemys scripta* individuals from Kehl, generated by ML-RELATE. Impressions of both habitats plus pictures of each species and the respective juveniles caught. Estimated log probability of data — Ln P(D), the mean likelihood of K (Delta K) and the number of simulated clusters for both *T. scripta* populations. Head and neck pattern in combination with plastral pattern of the hatchling 22367, showing intermediate morphological characters of both *Trachemys scripta* subspecies *Trachemys scripta elegans* and *Trachemys scripta scripta*.

Link: https://doi.org/10.3897/neobiota.82.87264.suppl1