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The feeding behaviour of the European pond turtle (*Emys orbicularis*, L. 1758) is not a threat for other endangered speciesCharlotte Ducotterd^{a, b, c, d, *}, Julien Crovadore^d, François Lefort^d, Antoine Guisan^{b, e}, Sylvain Ursenbacher^{f, g}, Jean-François Rubin^{c, d}^a Centre Emys, Association de Protection et Récupération des Tortues, Le Grand Pâquier 8, CH-1373, Chavornay, Switzerland^b Department of Ecology and Evolution, University of Lausanne, Biophore, Rue du Bugnon 21, CH-1015, Lausanne, Switzerland^c La Maison de la Rivière, Chemin du Boiron 2, CH-1131, Tolochenaz, Switzerland^d HEPIA, HES-SO, University of Applied Sciences and Arts Western Switzerland, 150 Route de Presinge, CH-1254, Jussy-Geneva, Switzerland^e Institute of Earth Surface Dynamics, University of Lausanne, Geopolis, CH-1015, Lausanne, Switzerland^f Department of Environmental Sciences, Section of Conservation Biology, University of Basel, St. Johannis-Vorstadt 10, CH-4056, Basel, Switzerland^g Info Fauna – Centre Suisse de Cartographie de la Faune (CSCF) and Centre de Coordination pour les Reptiles et les Amphibiens de Suisse (Karch), Bellevaux 51, CH-2000, Neuchâtel, Switzerland

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ABSTRACT

Molecular technologies, such as metabarcoding, have become powerful tools for conservation purposes. Here, we present a non-invasive study analyzing the diet of one population of European pond turtle (*Emys orbicularis*) during its whole activity period and of four other populations during the same period, based on faecal sample, and using for the first time on this species, a long metabarcoding approach. *Emys orbicularis* is an emblematic freshwater species of wetlands in Europe. In several countries, this species is endangered and, in Switzerland, *Emys orbicularis* is ranked as critically endangered on the Swiss Red List. A national conservation program was created to reintroduce this species and raised the question if this reintroduced species could be a threat for other endangered species. We developed a new method of long metabarcoding analysis, using universal PCR primers to determine prey species occurrence in the faeces. The analysis conducted on 174 faeces collected on 142 individuals revealed 1153 preys from 270 species. *Emys orbicularis* consumed plants throughout the year with a more diverse diet during the reproduction period (April–June). This study therefore not only determines precisely the omnivorous and opportunistic diet of the *Emys orbicularis*, but also shows that this species is not a threat to its environment, as 85.5% of the consumed species were not list on the Swiss Red List. Moreover, it also demonstrated that the genetic analyses of faeces could be an efficient tool to determine trophic interaction with a high level of precision, yielding promising perspectives for food web ecology.

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1. Introduction

The quantification of interactions and fluxes in food webs is of prime importance for the understanding of ecosystem functioning. Deciphering a diet composition provides the relative contribution of different food sources and therefore how predators (or consumers) can switch between resources. This knowledge is a crucial step for determining the potential impacts of a predator on its prey populations (Jedlicka et al., 2016; Krauel et al., 2018). Diet studies are essential as bases for conservation measures aiming to maintain optimal species interactions in the ecosystems (Soulé et al., 2003). This is particularly relevant in the current context of anthropogenic alterations of climate and ecosystems, which could probably result in the modification of the distribution, the availability and the abundance of food resources for many wild animal populations (Sanderson et al., 2002; Thuiller et al., 2001), thus leading to a severe biodiversity collapse (Global IPBES 2019 report). Conducting diet analyses can however be challenging with omnivorous predators feeding on a wide diversity of plant and animal species (De Barba et al., 2014). Furthermore, such predators are often opportunistic, and their feeding behaviour may vary temporally and/or spatially depending on prey availability, nutritional needs and environmental conditions; individuals of a predator species are also able to exhibit food choice variation (Williams et al., 2004; Kratina et al., 2012).

DNA metabarcoding is a powerful tool for conservation purpose, for studying food chains (King et al., 2008) and determine predator diet (Taberlet et al., 2012). DNA metabarcoding combined with next-generation sequencing (NGS) technologies (Shendure and Ji, 2008; Glenn, 2011) makes the taxonomic identification of DNAs present in soil, water, faecal, gut and stomach samples possible, by simultaneously sequencing in parallel thousands of DNA molecules corresponding to short DNA barcodes amplified by universal and/or specific primers (Valentini et al., 2009; Taberlet et al., 2012; De Barba et al., 2014). Nowadays, long metabarcoding provided much longer sequencing reads (Godwin et al., 2016), with longer markers increasing the ability to distinguish closely related species, allowing a higher taxonomic resolution (Singer et al., 2016). They advantageously allow the rapid and accurate identification and assignment of the taxonomic identity of preys, when the remains are degraded or when no hard parts are available.

Furthermore, samples, such as faeces, are obtained in ways that minimize interaction with animals and do not harm them (Pompanon et al., 2012; De Barba et al., 2014). Moreover, species-specific DNA sequences being easier to identify, these methods are among the most accurate approaches available to understand feeding behaviours in ecosystem (Valentini et al., 2009) and are also generally better at making species-level identifications compared to other biomarker methods such as stable isotopes, signature lipids and antigen detection (Symondson, 2002).

One emblematic species for which such new diet analysis approach is particularly beneficial is the European pond turtle (*Emys orbicularis*, L., 1758), a species occurring in the wetlands of Europe and North Africa and ranked as “near threatened” (NT) on the IUCN Red List. In Switzerland, this species is native and listed as “critically endangered” (CR) on the Swiss Red List (Monney and Meyer, 2005). In 1999, the *Emys* project was developed to protect and reintroduce this species in Switzerland (<http://www.karch.ch/karch/home/reptilien/reptilienarten-der-schweiz/europaische-sumpfschildkrote/wiederansiedlung-sumpfschildkrot.html>). Since then, three successful reintroductions took place in the country, but raised a critical question of whether this reintroduced species would feed on other threatened species, such as amphibian species (e.g. *Bufo bufo*, considered as VU in Switzerland, or *Rana temporaria*, NT) in their new environment and thus add a new threat to them.

Indeed, for a long time the diet composition of the European pond turtle remained unclear, since it was successively considered as carnivorous, often scavenger (Rollinat, 1934; Lebboroni and Chelazzi, 1991; Kotenko, 2000; Luiselli, 2017), sometimes vegetarian (Ficetola and De Bernardi, 2006), and finally, in more recent years, omnivorous (Ottonello et al., 2005, 2016, 2018; Çiçek and Ayaz, 2011). Up until now, methods used to determine food intake by *E. orbicularis* included direct observation and microscopic examinations (Ottonello et al., 2005, 2016, 2018; Çiçek and Ayaz, 2011). However, these techniques have several limitations, such as a loss of information and the difficulty in recognizing the type of prey (plants and animals) in the faeces. Here, we present a diet study conducted for the European pond turtle with the aim to support its reintroduction and conservation without harming other threatened species. To our knowledge, no metabarcoding study has yet explored the diet of this species and only a few metabarcoding studies analyzed the diet of reptiles, such as slow worm (Brown et al., 2012), Caribbean island lizard (Kartzinel and Pringle, 2015), red-eared slider and Reeve’s pond turtle (Koizumi et al., 2017). Although metabarcoding approach allows a precise determination of the species, this method does not allow the identification of the species stage (i.e. larva or adults, or roots, seeds and leaves). Moreover, the technique does not give information on the number or biomass of ingested items but only on the diversity of species consumed.

Currently, one of the drawbacks of metabarcoding approach is the limited length of the amplicons that are too short to allow assignment to the species level (Valentini et al., 2016). However, we recently developed a new method based on a long metabarcoding approach using primer redundancy and *de novo* assembly in order to efficiently and accurately identify taxon to the species level for plants, vertebrates and invertebrates present in faeces collected in the field (Ducotterd et al., submitted). In the present study, we aim to determine if *E. orbicularis* could be a threat for other endangered species (such as amphibians) by determining its diet using a universal and standardized method for a molecular and non-invasive diet assessment. We were interested assessing the overall variation in diet consumption over time, across locations and among individuals. In particular, we wanted to test whether differences in the diet of *E. orbicularis* exist between (i) month (temporal diet), (ii) populations (spatial diet), (iii) males and females and (iv) adults and juveniles; early studies suggest that the diet of juvenile and adult turtles from Emydidae family are different, with juvenile turtles being more carnivorous (Clark and Gibbons, 1969; Hart, 1983; Ottonello et al., 2005). Thus, we hypothesized that juveniles were more carnivorous than

adults. Ultimately, based on previous findings, we wanted to determine if threatened species could be found in the diet of the European pond turtle and in which individuals and populations.

2. Materials and methods

2.1. Study sites

Individuals of *E. orbicularis* were sampled in four different areas (Fig. 1), all sites are mature and stable ponds rich in vegetation and biodiversity. This type of habitats corresponds to the habitat used by the European pond turtle in the central and northern range of the species:

- (1) The natural reserve of Moulin de Vert (MDV; 46°10'46"N, 6°1'42"E) located in the canton of Geneva (Switzerland) downstream of the Verbois dam, on the left bank of the Rhone River. This habitat harbours the largest known population in Switzerland (about 180 adult individuals; S. Ursenbacher & M. Raemy, pers. comm.).
- (2) The natural reserve of Laconnex (LAC; 46°09'24"N, 6°01'46"E) located in the canton of Geneva (Switzerland). A population of about 150 individuals inhabit this area (C. Ducotterd, pers. obs.).
- (3) The natural reserve of Jussy (JUS; 46°15'04"N, 6°16'40"E) located in the canton of Geneva (Switzerland). In 2009, renaturation works were conducted, and first reintroduction took place in 2010; with a total of 52 ponds turtles being released there.

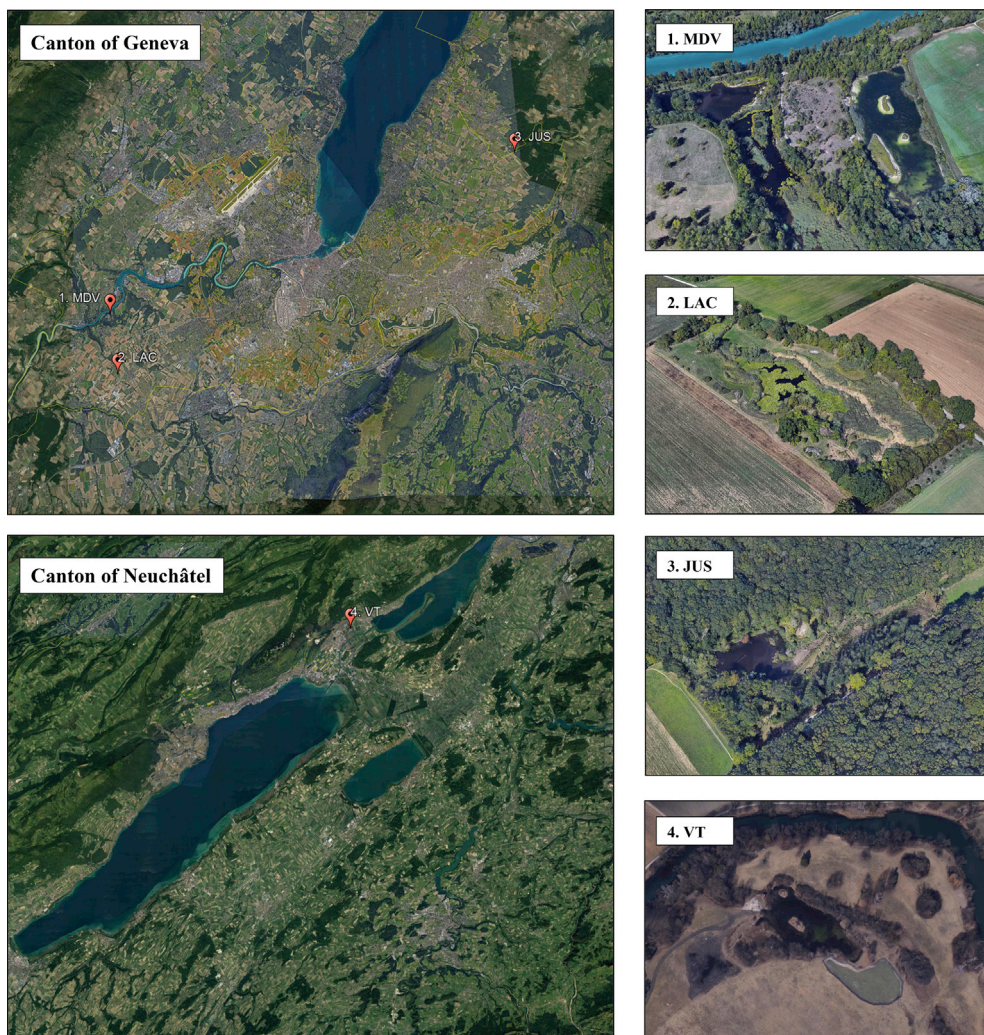


Fig. 1. Studied sites in cantons of Geneva and Neuchâtel (Switzerland). The diet of the European pond turtle was studied in four natural reserves (MDV = Moulin de Vert; LAC = Laconnex; JUS = Jussy and VT=La Vieille Thielle).

- (4) The natural reserve of La Vieille Thielle (VT; 47°2'57"N, 7°2'51"E) located in the canton of Neuchâtel (Switzerland). After some renaturation work conducted in 2009, the principal pond and the ancient oxbow lake of the Thielle River became the ideal habitat for the European pond turtle. Reintroduction took place in 2013, 2015 and 2019, a total of 27 European pond turtles were released until now.

2.2. Faecal samples collection

In order to determine their diet throughout their active season, European pond turtles were captured, with legal authorization, using conical fishing basket traps placed perpendicularly to the banks (Cadi, 2003) from April to September 2017 at MDV. Capture sessions took place each month and lasted for a week. Each trap was controlled every day and the captured pond turtles were placed in individual containers without water for the night, in order to collect faecal samples. Individuals were identified by notches made on marginal scales during previous monitoring studies, some others were not identified due to the absence of new monitoring studies (principally juveniles) and released at the exact location where they were captured. In order to compare diets at the same period in different location, European pond turtles were also captured using the same method during July 2017 in the JUS, LAC and VT sites. To prevent contamination of the samples, each container was cleaned with 10% bleach solution (NaOCl), followed by 70% denatured ethanol.

In total, 174 faecal samples from 142 individuals were collected in the field. All samples were stored in a plastic tube annotated with the individual's number, location and date of collection and then kept frozen at -80°C . In the laboratory, before grinding the samples with liquid nitrogen in order to proceed to DNA extraction, faeces were examined in order to determine visible prey items on the basis of their morphology, such as seeds, bones, shell, elytra, etc. This information was used as positive control for the metabarcoding approaches.

2.3. Metabarcoding approach

We developed a new method based on long DNA metabarcoding, primers redundancy and *de novo* assembly to identify different taxonomic group of organisms from complex diets (Ducotterd et al., submitted). We used previously published primers for the amplification of the large subunit of the ribulose-1,5-bisphosphate carboxylase gene (*rbcl*), the maturase K gene (*matK*), the 28S rRNA gene, the *trnL-trnF* gene region in plants, as well as a portion of the mitochondrial-encoded cytochrome oxidase subunit I (*COI* or *COX1*) gene in animals in order to amplify prey DNA extracted from faecal samples (Supplementary Material S1).

All PCR reactions were carried out in 25 μl reaction volumes, consisting of 5 μl MyTaq™ Reaction Buffer (Bioline GmbH, Germany), 2.5 μl of selected versatile primers (0.5 μM final concentration), 2 U of Bioline MyTaq™ DNA Polymerase (Bioline GmbH, Germany), 1 μl of DNA (concentrated at 10 $\text{ng}/\mu\text{l}$), and completed up to the final volume with ultrapure sterile water. Each PCR was run under the following conditions: an initial denaturation step at 95°C for 3 min, followed by 37 cycles of 95°C for 20 s, 52°C (annealing temperature for all primer sets except for the pairs *mCOLintF/jgHCO2198*, at 54°C , and *Tab c/Tab f* at 56°C) for 20 s and 72°C for 20 s, and terminated by a final extension step of 20 s at 72°C .

Then, 9 μl of PCR products amplified in triplicates with each distinctive primer pair were pooled per respective sample. Pooled PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) and diluted at 2 $\text{ng}/\mu\text{l}$ final concentration. Pooled DNA amplicons were fragmented to an average fragment size of 290 bp in AFA microtubes (Covaris, USA) using a S2 focused-ultrasonicator (Covaris) following our established protocol. Sequencing libraries were created using the TruSeq® Nano DNA HT Library Prep Kit (Illumina Inc., USA) following manufacturer Prep Guide. All samples were sequenced using two Illumina MiniSeq High Output run at 2 x 151 bp paired-end reads length, reaching a median sequencing depth of 106 Mb per sample.

Finally, cleaned sequencing reads were downloaded from the lab Illumina Basespace account. *De novo* assembly of amplicons per respective sample sequencing data was performed using the genome assembly open software "SPAdes 3.11" (Nurk et al., 2017), with the metagenome assembly option « metaSPAdes ». Sequences under 150 bp were deleted and resulting contigs files were then blasted on the server of the National Center for Biotechnology Information (NCBI) using the BLAST + suite of command line tools (Camacho et al., 2009), against the complete NCBI nucleotide (nr/nt) collection. Resulting sequences other than prokaryotes and fungi, and with identity $>97.6\%$ (this threshold was determined from the analyses of the mock communities and the captive feeding trials analyses) were selected to represent the prey consumed by the European pond turtles. More details about the whole methodology can be found in Ducotterd et al. (submitted).

2.4. Statistical analysis

All statistical analyses were run on the software RStudio (RStudio Team, 2015). In our statistical analyses, we selected non-parametric test as the sample distribution was asymmetric. In order to compare the distribution of plants, invertebrates and vertebrates in the diet of *E. orbicularis* and to determine differences with respect to sex (female or male), maturity (adult or juvenile), month (temporal diet), and sites (spatial diet), we used a proportion test, the Kruskal Wallis test and a factorial analysis (Multiple Correspondence Analysis-MCA) which used binary (presence/absence) data, in order to test the variation in

consumption of plants, vertebrates and invertebrates. Indeed, only the diversity of species and no information of the quantity (biomass and volume) of prey consumed are available with metabarcoding approaches.

More precisely in the MDV population, we studied differences by sex, periods and months in the number of species found in the diet. We measured the species richness within a group (sex, period or month). Additionally, we also calculated the dissimilarity in diet between groups, i.e. the β -diversity (change in composition).

The species richness, which the number of prey species, was simply the sum of our incidence (presence/absence) variable at the species level, based on the number of species detected in the diet of each turtle.

Then to determine the β -diversity, which is a measure of dissimilitude between observations, we used the Jaccard index, which is the most common index used for assessment of β -diversity (Jaccard, 1912; Gianni et al., 2011; Ricardo and Francisco, 2011) and defined as follows:

$$S^j = \frac{a}{a + b + c}$$

where a is the overall richness, b is the number of species which appeared only in sample B and c is the number of species which appeared only in the sample C. Jaccard's dissimilarity index is $1 - S^j$. This index ranges between 0 and 1; if the index equals to 0 then all species observed appear in both samples, if the index is 1, and, all species are different. The β -diversity computes the distance in terms of species in the diet between two observations; we compared pairs for sex, pairs of Female-Female, Male-Male and Male-Female, as well as for period combinations for both April–June, both from July–September and one from each period. For that, we computed statistics for sets of individuals belonging to the same category and for those belonging to different categories.

To compare statistically the distribution of prey species (species richness) by sex and by period (April–June vs. July–September), we did a univariate analysis using a non-parametric test (Wilcoxon signed-rank test). Furthermore, in order to compare prey species between months, we used a Kruskal-Wallis rank sum test comparing all month by pairs. Finally, Dunn's tests were used to compare for the number of species by months and to determine difference in β -diversity between sex, period and months.

3. Results

In 174 faeces from 142 individuals collected on the field, a total of 1153 preys were consumed from 270 different OTU, of which 86.1% could identified to species level, while the others were determined only at the genus/family level (Supplementary Material S2). In our sample, pseudo-replications are low as very few turtles are observed for multiple months. More in details, we recaptured twice 13 individuals and four times 2 individuals. Thus, it would not be possible to take into account individual effect as we could with a balanced panel (with all the individual replicated on each time period). Moreover, note that as the juvenile turtle is missing an identification number, it is impossible to track which juvenile turtle has been recaptured. Moreover, the fact that p-values are far below the 5% threshold strengthens the idea that rare pseudo-replications would not affect the conclusion of this paper.

3.1. Moulin de Vert (MDV) population - Temporal diet assessment

In the natural reserve of MDV, 146 faecal samples were collected from 114 individuals (83 marked and 31 non-marked individuals). Samples were collected on 71 females, 45 males (females were overrepresented), and 28 juveniles between April and September (80.6% adults and 19.4% juveniles). The DNA sequencing of all these faecal samples revealed that 97% of the faeces contained plants, 81% macro-invertebrates and 15% vertebrates.

Differences in the consumption of plants, vertebrates and invertebrates were not significant between females and males (Kruskal Wallis test; plant: p-value = 0.680; vertebrates: p-value = 0.444; invertebrates: p-value = 0.526; Fig. 2a).

Regarding maturity, no significant difference was found in the proportion of plants (Kruskal Wallis test; p-value = 0.319), invertebrates (Kruskal Wallis test; p-value = 0.774) and vertebrates (Kruskal Wallis test; p-value = 0.820) in the regime of juveniles versus adults (Fig. 2b).

Our results showed a significant difference in vertebrate consumption between the reproduction period – April to June, corresponding to mating and egg laying period – and the post reproduction period – July to September – with higher consumption during the reproduction period (Kruskal Wallis test; p-value = 0.008). For invertebrates, the difference was not significant (Kruskal Wallis test; p-value = 0.139) and the proportion of plant ingestion did not change between the two periods (p-value = 0.293). In other words, consumption of plant and invertebrates were similar through the whole activity period, only the consumption of vertebrates decreased in summer (Fig. 2c).

The Multiple Correspondence Analysis (MCA) confirmed this relationship (see Fig. 3a and b). The European pond turtle seems to diversify its diet during the reproduction and egg laying period by eating more vertebrates.

3.1.1. Number of species – species richness

Overall, the number of species in the diet went from 1 to 24 with a median of 6 and a mean slightly higher than 6.9 (Fig. 4a; Table 1). We noticed that the number of species in the diet was lower for the post-reproduction period (July–September) with

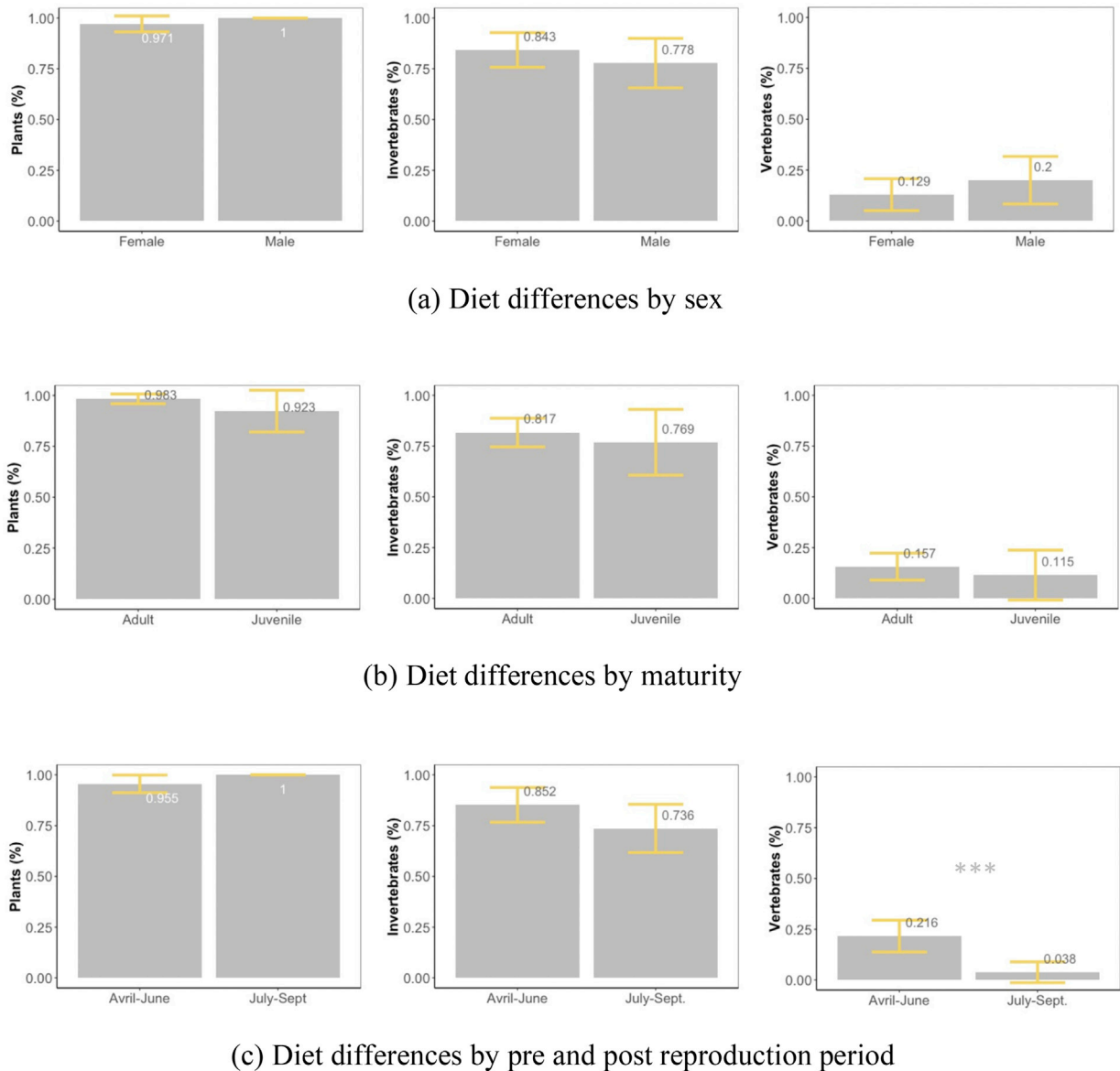


Fig. 2. Temporal diet (April to September) of the European pond turtle population of the natural reserve of Moulin de Vert (MDV; Geneva, Switzerland). (a) Difference between males and females in plants, invertebrates and vertebrates' consumption, (b) difference between adults and juveniles in diet and (c) variation in diet between the pre-reproduction period (April–June) and the post-reproduction period.

a mean of 3.4, a median of 3.0 and a maximum of only 7, while for the reproduction period (April–June), the mean and median were 9.0 with a maximum of 24 (Fig. 4b). The period was statistically significant, influencing the number of species consumed (p -value < 0.001; Fig. 5a). The mean and the median were higher for males (7.0 and 7.6 respectively) compared to females (5.0 and 6.5 respectively). However, this difference may be the result of an over-representation of males during April to June (35 observations) compared to July to September (only 10 observations; Fig. 4c).

Using a non-parametric test, we cannot say that sex influenced the number of species consumed (Wilcoxon signed-rank test; p -value = 0.068, Fig. 5a). Furthermore, the dietary habits of the turtles were clearly split in two periods: April–June (pre-reproduction) and July–September (post-reproduction). The comparison of all months by pairs reflected this fact as all statistically significant differences (Kruskal-Wallis rank sum test; p -value < 0.001) were between months from April to June with months from July to September (Fig. 5a).

3.1.2. β -diversity

The β -diversity demonstrated that the difference by period was highly significant, which corroborated our previous findings (Dunn's test; p -value < 0.001) with 95.5% of the species consumed between the reproduction period (April–June) and the other months (July–September) (β -diversity = 0.955, Table 1 and Fig. 5b).

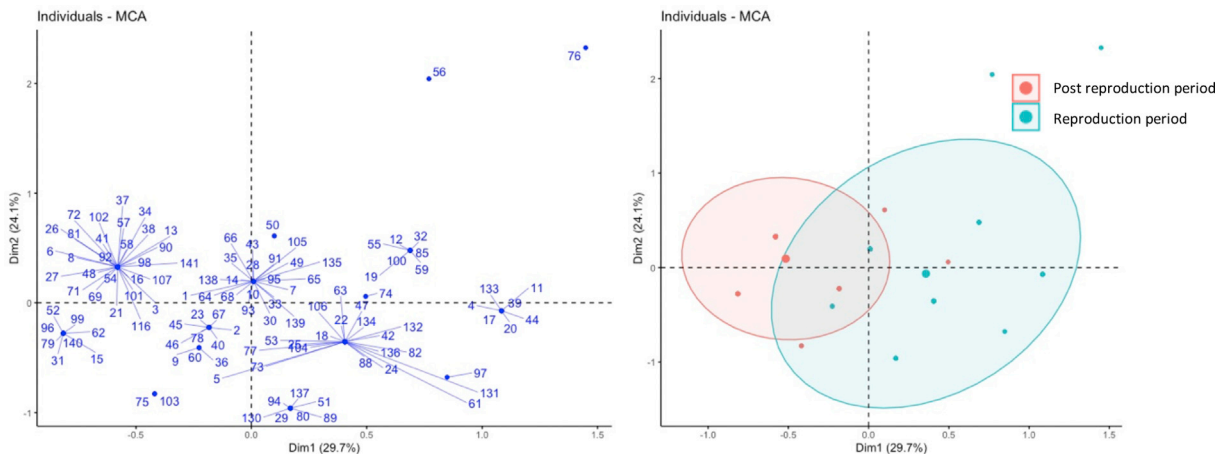


Fig. 3. Multiple Correspondence Analysis (MCA) of the composition (plants, invertebrates and vertebrates) of faeces obtained from European pond turtle in the natural reserve of Moulin de Vert (MDV; Canton of Geneva, Switzerland). (a) Individuals samples (blue points) demonstrated a massive overlap of two clusters of individuals (numbers connected by segments). (b) demonstrated the same clustering without labels ID but with a color code (Reproduction period between April–June). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

β -diversity sex showed no statistical difference between the distribution of Female–Female and Male–Male pairs (Dunn's test; p -value = 0.095). However, the pairs Female–Male were statistically different (Dunn's test; p -value < 0.001) showing that indeed the dissimilarity is larger between female and male with 90.5% (β -diversity = 0.905) of the species consumed being different (Table 1 and Fig. 5b).

3.2. Comparison between four populations - Spatial diet assessment

In July 2017, samples were collected in four different populations of *E. orbicularis*. During this period, 56 faecal samples were collected on 56 individuals represented by 30 females, 13 males and 13 juveniles (76.8% adults and 23.2% juveniles). The metabarcoding DNA sequencing of the 56 faecal samples revealed that 100% faeces contained plants, 12.5% vertebrates and 85.7% macro-invertebrates (Fig. 6). Unfortunately, only two European pond turtles were captured in VT due to the very low number of turtles present in this population ($n = 18$). Therefore, the number of samples was too small to allow a relevant statistical analysis on this population.

No significant differences were found between sites (MDV, LAC and JUS) regarding consumption of the three groups of organisms evaluated (Kruskal Wallis test, p -value = 0.220).

3.3. Endangered species consumption?

Three species of amphibian were found through the metabarcoding DNA sequencing of faeces; the common toad (*Bufo bufo*, L., 1758; found in 13 occurrences), the green frog (*Pelophylax lessonae*, Camerano, 1882; 1 occurrence) and one sample containing bones of which DNA analysis allowed us to determine the common frog (*Rana temporaria*, L., 1758) (see Table 1). Some other species ranked as near threatened or vulnerable in the Swiss Red Lists were also consumed by the European pond turtles, especially a moss, *Pleurochaete squarrosa* (Limpr., 1888; 20 occurrences), a fish *Cyprinus carpio* (L., 1758; 8 occurrences) an odonatan, *Gomphus pulchellus* (Selys, 1840; 4 occurrences), and a slug *Deroceras laeve* (Müller, 1774; 4 occurrences). More anecdotally, we found the presence of one odonatan species, *Coenagrion pulchellum* (Vander Linden, 1825), a butterfly species, *Polyphoca ridens* (Fabricius, 1787), a caddisfly, *Limnephilus vittatus* (Fabricius, 1798), a snail *Zonitoides nitidus* (Müller, 1774) and a plant *Typha angustifolia* (L. 1753), which were also considered threatened on the Swiss Red Lists. Some plants, such as *Nymphaea alba* (L., 1753) and *Utricularia australis* (R. Br., 1810) were more frequently found, with 90 and 18 occurrences, respectively (Table 1). Many undigested seeds were also found in the faecal samples and species were determined based on their morphology and then confirmed with our metabarcoding approaches. These results precisely showed that 13 endangered species were consumed by the European pond turtle. Therefore, its diet was composed of the 270 different species, meaning that 85.5% of them were not considered as threatened on the Swiss Red Lists or were listed as “data insufficient”.

4. Discussion

We presented results from a new molecular approach to assess the diet of a reintroduced species to better understand the potential impact of its feeding habits on other species. Using long metabarcoding approach to determine dietary composition and richness in a sample of any kind (water, faeces, gut content, soil, etc.) allowed us to widen the pre-existing knowledge of

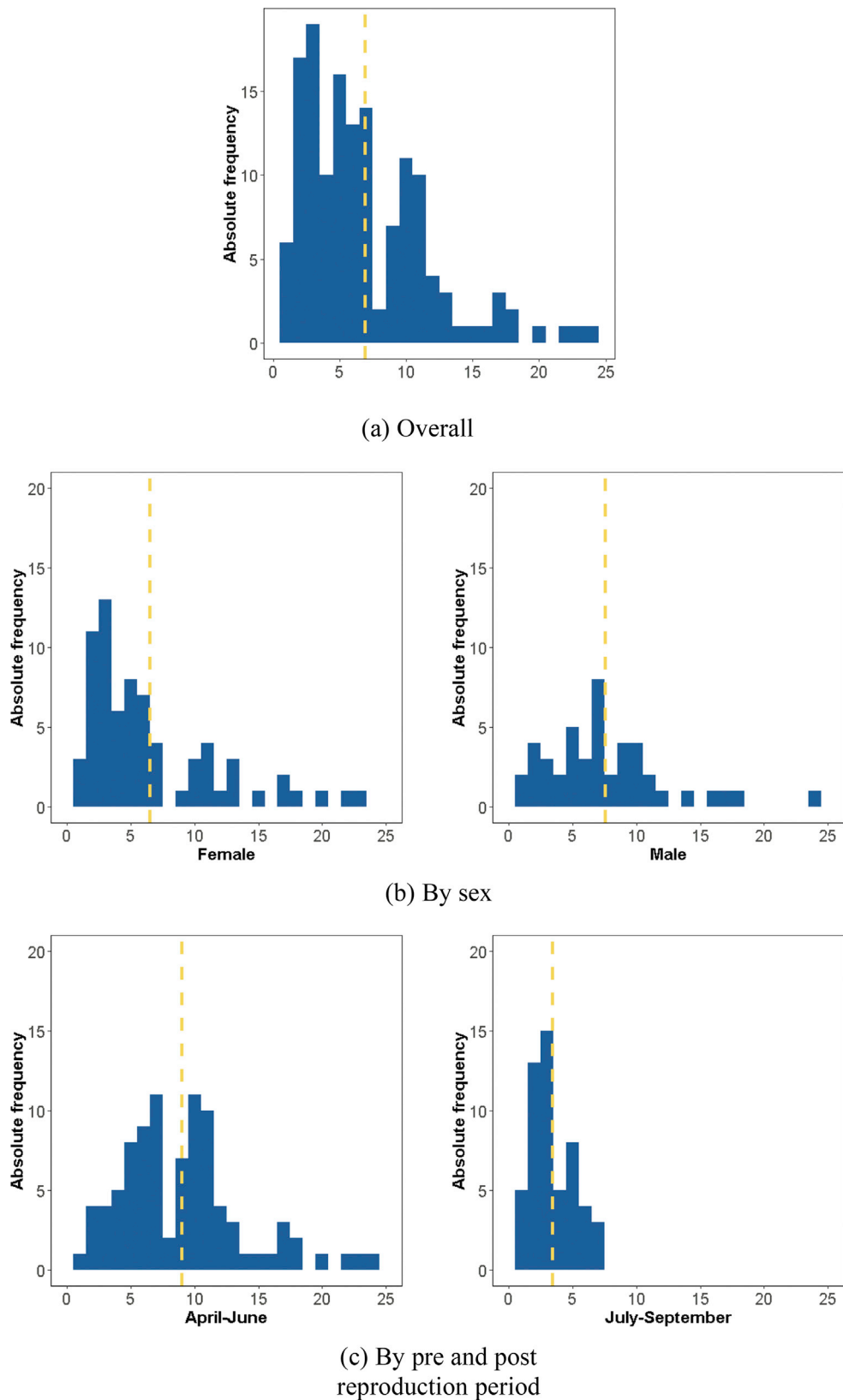
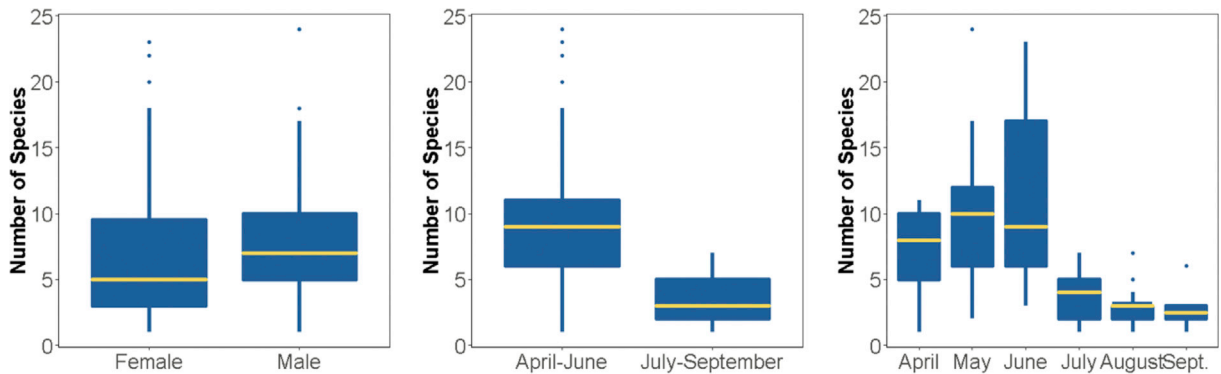


Fig. 4. Distribution of the number of species consumed of the European pond turtle (a) overall; (b) separated by sexes; and (c) differences between the pre- and post-reproduction period.

Table 1

Summary statistics of the number of species, the β -diversity of the diet of the European pond turtle during its whole activity period (April to September) in the population of Moulin de Vert (Geneva, Switzerland).

	Mean	Median	SD	Min.	Max.
Number of species (richness)					
Overall	6.944	6	4.792	1	24
Sex					
Female	6.535	5	5.253	1	23
Male	7.578	7	4.76	1	24
Period					
April–June	9	9	4.823	1	24
July–September	3.415	3	1.669	1	7
β-diversity (Jaccard)					
Overall	0.907	0.933	0.115	0	1
Sex					
Female, Female	0.897	0.933	0.129	0	1
Male, Male	0.89	0.917	0.114	0	1
Male, Female	0.905	0.929	0.12	0	1
Period					
Both April–June	0.901	0.917	0.088	0	1
Both July–September	0.756	0.8	0.148	0	1
Not in the same period	0.955	1	0.081	0	1



(a) Number of prey species in the diet

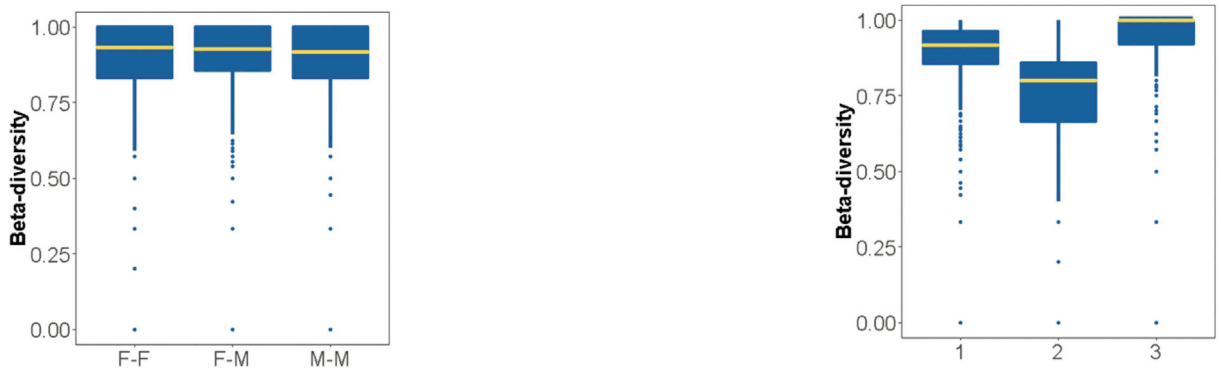
(b) β -diversity (Jaccard index)

Fig. 5. Box plot of the number of species (species richness) and β -diversity (Jaccard index) in the diet of the European pond turtle during its whole activity period (April–September). (a) Difference in number of species found between male and female, pre- and post-reproduction period and between each month, (b) β -diversity in the diet by sex and pre- and post-reproduction period (F = female, M = male, 1 = pairs from April–June; 2 = pairs from July–September and 3 = pairs with one observation from April–June and the other from July–September).

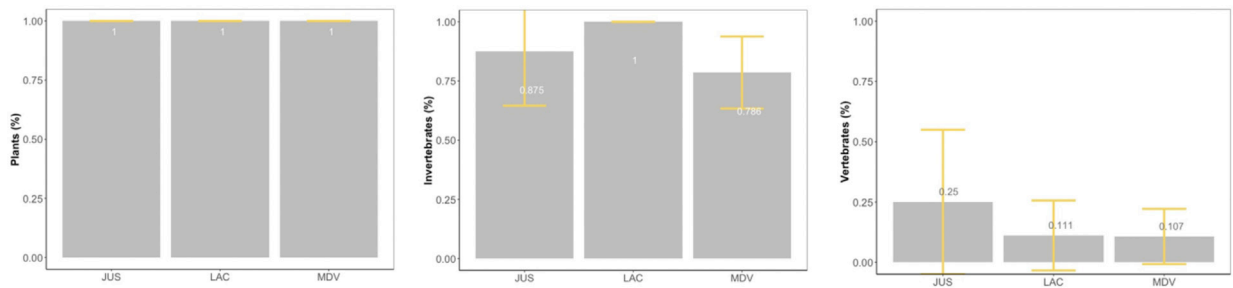


Fig. 6. Spatial variation in the diet of the European pond turtle in three different population of Switzerland (JUS = Jussy; LAC = Laconnex and MDV = Moulin de Vert, see Fig. 1) during the month of July. Proportion of plants, invertebrates and vertebrates consumed among populations.

the studied species' ecology and behaviour. Indeed, the higher level of ingested species richness found with the new molecular approach compared to histological analyses of the same sample, corroborated the result of other studies on different species (Soininen et al., 2015; Ando et al., 2013). Our results demonstrated a great precision, since 86.1% of the preys were identified to the species level and revealed a large species richness in the diet of the European pond turtle. Previous studies on the diet of this species, which were only made via direct observation or using microscope (Ottonello et al., 2005, 2016, 2018; Çiçek and Ayaz, 2011), only yielded the identification of preys, in most cases to the order level, or in some cases to the family level, and very often, plants were defined as “unidentified organic matter” or “plant fragments”. This resulted in a huge loss of ecological information.

In the present study, we revealed the presence of 1153 prey items from 270 different species of vertebrates, invertebrates and plants, consumed by the European pond turtle. Consequently, assessing diets only via the visual identification of ingested prey results in a wide underestimation of taxonomic diversity. The integration of new metabarcoding approaches is therefore essential to provide a more precise knowledge of diet and feeding strategy, even if this approach does not allow the evaluation of the amount of each prey.

Our results show that the European pond turtle feeding strategy in Switzerland follows an opportunistic and omnivorous pattern. Moreover, almost all sampled turtles consumed plants in their diet and throughout their activity period, suggesting that aquatic plants are a key component of the diet of *E. orbicularis*, in contradiction with previous studies which stated that plant matters were accidentally ingested together with animal preys, and did not represent a primary food item (Lindeman, 1996). This actually addresses the question whether the plant fragments found in our samples could come from animal preys, as some of these are plant consumers. In this regard, the DNA present in faecal sample is usually degraded (Deagle et al., 2006), meaning that food items eaten by preys, itself eaten by the predator, were degraded twice, making unlikely the possible detection of plant DNA ingested by the preys. Moreover, a large proportion (97.9%) of the analyzed faeces contained plants. Consequently, our findings are corroborating previous results (Ficetola and De Bernardi, 2006; Ottonello et al., 2016) showing the presence of large pieces of plant matter such as leaves from *Typha* or *Phragmites* and seeds from *Nymphaea*. These results likely support that the European pond turtles ingested these plants voluntarily as food item (Ayres et al., 2010).

Concerning the difference between adults and juveniles in the diet of the Emydid turtle family, previous studies (*Trachemys scripta elegans*: Clark and Gibbons, 1969; Hart, 1983; *Emys orbicularis*: Ottonello et al., 2005) suggested that adult turtles feed more frequently on plants than juveniles, and proposed that turtles shift to a more herbivorous diet as they grow. Surprisingly, our results did not corroborate these views and rather demonstrate a lack of difference in plant consumption between juveniles and adults. However, our analyses are based on the number of species and not on proportions of ingested items or their volumes or biomass. Thus, the morphological and molecular methods should be considered as complementary approaches in order to determine both species and the volume or biomass consumed. In the case of juveniles, their faeces are extremely small and therefore visual determination of prey items are very difficult or impossible.

Moreover, previous studies reported that the consumption of plants increased during the post breeding period, suggesting a diet shift throughout the year (Ottonello et al., 2005; Ayres et al., 2010). Yet, our study demonstrated that the plant consumption did not change through the year. Indeed, we demonstrated that the number of species (species richness) was larger from April to June, meaning that plants species were not just replaced by invertebrates and vertebrates, but that the diet was indeed more varied in spring. Furthermore, we showed that the dissimilarity between females and males was strongly significant, meaning that both sexes consumed different species. Regarding the period, the average Jaccard β -diversity was 0.955, meaning that the European pond turtle has a completely different diet between April–June and July–September with on average 95.5% difference in the species being consumed by turtles between periods. Therefore, this result confirms the opportunistic and omnivorous diet of the European pond turtle in Switzerland.

We demonstrated that 97.9% of the faeces contained plants. This result combined with those of species turnover (β -diversity) suggests that the plant species being consumed varied greatly through time, meaning that plant-based regime is not constant through months, but most certainly evolves over time.

Another significant change in diet was related to vertebrate's consumption which was higher during the reproduction period (April–June). We can hypothesize that the European pond turtle consumes less vertebrates after the reproduction period due to the fact that its energy need, and thus its hunting activity, is reduced.

The diversity in diet might therefore be due to the temporal availability of preys, as some species are only present in the pond for short periods. Amphibians are, for instance, dense in spring with *Bufo bufo* and *Rana temporaria* breeding early, whereas tadpoles remain available until the beginning of summer, except for *Pelophylax* species. The European pond turtle thus seems to behave opportunistically and to target preys that are the easiest to obtain, in congruence with the optimal foraging theory (MacArthur and Pianka, 1966). Observing the abundance of species present in the environment through months would allow observing a potential variation of availability between the April–June and July–September periods.

In our study, the ponds inhabited by the European pond turtle are mature and stable and correspond to the habitat in the central and northern range of the species, therefore we could hypothesize that the diet and trophic niche would be very similar to the population in Switzerland. However, as the European pond turtle seems to have an opportunistic behavior, the species can locally eat completely different prey depending on their microhabitat.

In future studies, comparison with subpopulations of the same species, such as *E. orbicularis galloitalica*, which live in rivers in Corsica, or *E. orbicularis persica* from Jelilabad, Azerbaijan, which is considered as carnivorous (Luiselli, 2017) or with populations living in Mediterranean ponds, with high seasonal variations in water level and water temperature, would provide additional insights into the general ecology of this species across its range.

4.1. Is the European pond turtle a threat for other endangered species?

The European pond turtle is considered a vulnerable species at the European level and has even disappeared in numerous regions. Consequently, several reintroductions occurred in Western Europe (Fritz and Chiari, 2013). One of the crucial questions before any reintroduction has always related to its potential danger to other threatened species.

Our study, however, demonstrates that the European pond turtle mainly eats plants. Regarding the consumption of threatened plants such as *Nymphaea alba* (NT) and *Utricularia australis* (NT), we could deduce from the high quantity of seeds, which were determined by morphological observations and DNA analyses, present in faeces that the European pond turtle consumed mostly their fruits (see Table 2). This turtle species might therefore also participate in the dissemination of their seeds, together with other turtle species (Kimmons and Moll, 2010; Padgett et al., 2018). Moreover, *Nymphaea* seeds were also shown to germinate better after transiting in the digestive system of the European pond turtle (Calvino-Cancela et al., 2007; Ayres et al., 2010). Regarding the consumption of threatened invertebrates and vertebrates, some of these were only occasionally eaten. *Bufo bufo* was the most consumed vertebrates (present in 13 out of 174 samples), and only during the European pond turtle reproduction period, which corresponds to the presence of tadpoles in the pond (Table 2). Unfortunately, one of the limitations of metabarcoding approaches is related to the status of the prey; indeed, it is impossible to determine whether adults, juveniles, larva, or/and eggs were consumed, and if individuals were dead or alive when eaten. In our case, we could only hypothesize that the European pond turtle consumed tadpoles in spring. To conclude, the variety of preys consumed by the European pond turtle strongly suggests that this species is an opportunistic hunter and its impact on other endangered species is rather marginal.

Table 2
Threatened species found in the diet of the European pond turtle in Switzerland.

Phylum	Class	Order	Family	Genus	Species	Statut on the Swiss Red List	Found in x samples on 174
Chordata	Amphibia	Anura	Bufo	<i>Bufo</i>	<i>Bufo bufo</i>	VU	13
Chordata	Amphibia	Anura	Ranidae	<i>Rana</i>	<i>Rana temporaria</i>	NT	1
Chordata	Amphibia	Anura	Ranidae	<i>Pelophylax</i>	<i>Pelophylax lessone</i>	NT	1
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	<i>Cyprinus</i>	<i>Cyprinus carpio</i>	NT	8
Plantae	Spermatophyta	Nymphaeales	Nymphaeaceae	<i>Nymphaea</i>	<i>Nymphaea alba</i>	NT	90
Plantae	Equisetopsida	Lamiales	Lentibulariaceae	<i>Utricularia</i>	<i>Utricularia australis</i>	NT	18
Plantae	Tracheophyta	Poales	Thypaceae	<i>Typha</i>	<i>Typha angustifolia</i>	NT	3
Plantae	Equisetopsida	Pottiales	Pottiaceae	<i>Pleurochaete</i>	<i>Pleurochaete squarrosa</i>	VU	20
Mollusca	Gastropoda	Stylommatophora	Agriolimacinae	<i>Deroceras</i>	<i>Deroceras laeve</i>	NT	4
Mollusca	Gastropoda	Stylommatophora	Gastrodontidae	<i>Zonitoides</i>	<i>Zonitoides nitidus</i>	NT	1
Arthropoda	Insecta	Trichoptera	Limnephilidae	<i>Limnephilus</i>	<i>Limnephilus vittatus</i>	VU	1
Arthropoda	Insecta	Lepidoptera	Drepanidae	<i>Polyploca</i>	<i>Polyploca ridens</i>	VU	1
Arthropoda	Insecta	Odonata	Coenagrionidae	<i>Coenagrion</i>	<i>Coenagrion pulchellum</i>	NT	3
Arthropoda	Insecta	Odonata	Aeshnoidea	<i>Gomphus</i>	<i>Gomphus pulchellus</i>	VU	4

5. Conclusion

Our study demonstrated the use of metabarcoding as a powerful tool for conservation purpose, allowing to provide precise answers to ecological questions about specific diets. As the European pond turtle benefits from a national conservation program in Switzerland, the key question was to determine if its reintroduction in new locations might threaten other endangered species. The answer provided by long metabarcoding rather suggests a very marginal impact. Using metabarcoding further made it possible to considerably improve our understanding of the feeding behaviour of the European pond turtle and the diversity of preys consumed, at a level never reached before. Our approach and findings also offer a great perspective in future studies of food web and trophic interactions. As next possible steps, comparisons of these results with other *Emys* populations living in different environment would greatly improve knowledge of the whole genus, found in very diverse environments with diverse food opportunities.

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Author contribution

CD, SU and JFR designed the study, CD conducted the sampling, the laboratory and the bioinformatics analyses, JC set up the methodology (primers selection and design, sequencing and bioinformatics analyses), JC and FL supervised the laboratory work. CD wrote the manuscript and the other coauthors revised it.

Data accessibility

All raw sequencing reads for 182 metabarcodes were registered in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the Bioproject accession PRJNA546135.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gecco.2020.e01133>.

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