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Impact of invading species on biodiversity: Diet study of the green whip snake's (*Hierophis viridiflavus*, L. 1789) in Switzerland

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ABSTRACT

Next-generation sequencing is increasingly used in conservation biology to resolve complex interactions between species, either diet or gut parasites studies. We applied a recent long metabarcoding method to elucidate the green whip snake's (*Hierophis viridiflavus*) prey consumption based on DNA extracted from stomach contents. Illegally introduced in Canton of Vaud (Switzerland), three populations of the green whip snake have strongly developed in two regions, East (Chablais) and North. We suspect that this introduced species is threatening part of the local herpetofauna, especially the Asp viper and the Western green lizard in this region. Consequently, an extermination program has been implemented from 2016 to mitigate *Hierophis viridiflavus* expansion and its impact arising from its generalist diet. Stomach contents of 94 individuals removed from introduction sites were analysed by long metabarcoding. Our study revealed the consumption of 67 prey belonging to 9 species, primarily small mammals and reptiles. The recurrent presence of two parasitic nematodes was also discovered. Although cannibalistic behaviour could not be highlighted with this approach, a scavenging behaviour was suspected based on the presence of an insect used in forensic entomology (*Calliphora vicina*). These results confirm the opportunistic feeding behaviour of *Hierophis viridiflavus* and its ability to predate on threatened species. Although 86.6 % of preys were not listed on the Swiss Red List, the impact on the Asp viper population can be important (up to 20 % of consumed preys) and could partially explain its strong decline.

1. Introduction

According to the concept of ecological niche, ther species' persistence within an ecosystem is explained by their habitat requirements and their own functional role in this ecosystem. Both the biocoenosis and the biotope dynamically interact each other in time and space, ultimately shaping ecosystems (Polechová et Storch, 2008). Utilization of resources, such as food supply and microhabitat occupation, induces competition and trade-offs between and among species. In natural habitats, many species coexist,

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including closely related ones, partly due to resources partitioning and differing requirements of individuals. Otherwise, when cohabitation is not possible, the least competitive species would need to adapt by moving or tend towards extinction (Pocheville, 2015). This concept has led to a better understanding of the ecosystems' functioning and can help predict population dynamics and the evolution of communities.

Depending on the pressure exerted on the ecosystem, a species can become dominant and generate important biodiversity losses or, on the contrary, become rapidly vulnerable to the slightest ecological disturbance (Clavel et al., 2011). One of the main challenges facing conservation today is biological invasion, mainly caused by the human-induced introduction of non-native species outside their range, either intentionally or accidentally (Lévêque et al., 2010). A species is considered invasive when it significantly increases its initial population size and extends its range, causing environmental, economic, or health problems (Pascal et al., 2011). To better control and prevent biological invasions, it is necessary to study the response of non-indigenous organisms to their new environment and the resulting interactions, such as food webs alterations (Alpert, 2006).

Indeed, the feeding behaviour of a species has important impacts on its environment. In functional ecology, a distinction is often made between dietary generalists and specialists, and this distinction plays a major role in conservation and evolution. An individual with a generalist feeding behaviour will have a broad, more or less diverse, food spectrum, depending on seasonal needs and environmental opportunities. Specialist predators, on the other hand, will have a narrower food spectrum and are generally considered as more vulnerable (Machovsky-Capuska et al., 2016). Therefore, specialists are more prone to extinction, while generalists are assumed to be more resilient, having a greater capacity of adaptation and dispersion, and thus becoming more easily invasive. While this concept is a useful categorization, species' diets can fluctuate between these two behaviours (Ducatez et al., 2015).

In this context, unravel the diet composition of introduced species is essential as they can profoundly affect the local fauna and flora. Such feeding behaviour studies will allow us to determine the extent of their impact on the ecology of local species. This knowledge can promote conservation decisions and action plans to be implemented for restoration of ecosystem balance (Barba et al., 2014).

DNA metabarcoding using next-generation sequencing (NGS) represents a powerful analytical tool widely used for ecological and conservation applications. Through this approach, multiple taxa present in various substrates, such as soil, water, hairs, feces or stomach contents, can now be analysed in parallel. The use of such a method is particularly relevant for the resolution of complex samples in diet assessment within complicated food webs, as seen in omnivorous species or species difficult to observe under natural conditions (Barba et al., 2014). This technique enables the simultaneous identification of large taxonomic groups of prey that may derive from a variety of predator samples, even in the case of rare food items' ingestion. Whether through the analysis of feces, gut contents or regurgitation, high-throughput sequencing allows to resolve the dietary spectrum of species with an increased precision (Barbato et al., 2019). Nonetheless, some limitations of this approach are still difficult to overcome.

Indeed, the results are directly influenced by the selection of molecular markers and their different taxon-contingent affinities according to amplification primers specificity. As collected samples can be highly heterogeneous, large differences in the quality and quantity of extracted DNA may affect the ability to identify any prey (da Silva et al., 2019). Besides, possible contamination during sample processing may occur, not to mention that the quality and exhaustiveness of reference databases can be a stringent limiting factor (Forin-Wiart et al., 2018).

Furthermore, DNA metabarcoding studies are mostly based on short DNA fragments, which restricts their taxonomic resolution's accuracy. Nowadays, this limitation is being increasingly overcome by the use of long reads sequencing technologies and genome assembly softwares, which produce longer contigs. Thereby, such approaches as the long metabarcoding method developed by (Ducotterd et al., 2020), the use of universal primers and *de novo* assembly of short DNA reads allow the formation of long contigs and the accurate specific determination of ingested prey, even on degraded material (Ducotterd et al., 2020, 2021)

The green whip snake (*Hierophis viridiflavus*, L. 1789) is a Colubrid snake widespread in Western Europe. This diurnal, thermophilic species is known to be a terrestrial racer with high energy requirements represented by two phenotypically distinct subspecies (Lelièvre et al., 2012a). The nominal morphotype, *Hierophis viridiflavus subsp. viridiflavus* has a black and yellow phenotype and is found in northwestern Italy, Sardinia, France and Spain, whereas *Hierophis viridiflavus subsp. carbonarius,* the melanic morphotype, occurs throughout Italy (except the northwestern part and including Sicily), in Slovenia and Croatia. This morphotype is thought to have emerged after a vicariant speciation event (Racca et al., 2020), hypothetically caused by a combination of glacial, tectonic and eustatic factors during the Pleistocene (Mezzasalma et al., 2015). Although both morphotypes have been commonly treated in the literature as subspecies, Mezzasalma et al. (2015) proposed to elevate *H. v. subsp. carbonarius* to species following morphological and phylogenetic analyses and to name it *Hierophis carbonarius* (Bonaparte, 1832). Nevertheless, a study conducted by Gramolini et al. (2018) found that gene flow occurred within an area where the two taxa came into contact, refuting the recent assignment of *Hierophis carbonarius*. Since then, the two morphotypes are still considered subspecies (Racca et al., 2020; Gramolini et al., 2018).

In Switzerland, *H. v. subsp. viridiflavus* is naturally distributed in the canton of Geneva, while *H. v. subsp. carbonarius* is present southern of the Alps (Zuffi, 2008). However, a few decades ago, individuals of both subspecies were illegally introduced in three places of the canton of Vaud, and the populations expanded during the last decade. The *viridiflavus* morphotype was released in the North of the canton of Vaud, where the Asp viper is already threatened, at two sites near Yverdon, while the *carbonarius* morphotype was introduced into the Chablais Vaudois (East) where it has proliferated. Consequently, the consulting firm Hintermann & Weber SA was commissioned by the local authorities via info-fauna karch to survey the reptiles present in these areas and to capture and euthanize the green whip snakes found there between 2016 and 2020. During these commissioned field trips, individuals were observed and their distribution determined to evaluate their expansion and, subsequently, the impact of ongoing control efforts.

Although considered a generalist species with a varied food spectrum consisting of mammals, reptiles, birds and amphibians (Lelièvre et al., 2012b), Switzerland's green whip snake's diet has not yet been studied. Regarding its foraging strategies, the

consumption of snakes demonstrates ophiophageous behaviour. Ophiophagy is a known snake feeding behaviour widespread in colubrid species, with several documented cases of consumption of other Colubrids, on Vipers or even cannibalism (Capula et al., 2014). Considering this feeding behaviour of *Hierophis viridiflavus* and its coexistence with endangered species of reptiles in the canton of Vaud, it was essential to study the diet of these introduced populations. For this purpose, a long metabarcoding method (Ducotterd et al., 2020, 2021) was used in this study to overcome the lack of taxonomic resolution accuracy, known to be the main limitation of this approach. The main aim of this study was to assess whether these introduced populations induced competition or predation on local communities according to sites and time, with a particular interest in threatened herpetofauna. Indeed, in the case of introduced species, it is crucial to assess the extent of their impact on local species communities and fight their expansion at an early stage, before it is too late to react.

2. Materials and methods

2.1. Study sites

Hierophis viridiflavus is present in dry vegetated areas, whether in shrubby sites, open forests or rural gardens. All individuals were collected from three sites of the canton of Vaud (Switzerland), in the two regions of introduction, the North of the canton and the Chablais region.

(1) Jura 1 (about 46.8°N, 6.6°E), located in the North of Vaud, where the presence of the Asp viper is known.
(2) Jura 2 (46.8°N, 6°7E), also located in the North of Vaud, where the Asp viper is present and the Western green lizard has been introduced. The distance between both Jura sites is ca. 10 km.
(3) Chablais (46.3°N, 7.0°E), located in the Chablais region, hosts the largest population (over 60 individuals were observed in 2016).

2.2. Stomach content collection

Hierophis viridiflavus individuals were collected between 2016 and 2020 in the three sites with a similar sampling effort throughout the Northern part of Vaud (Jura 1 and 2) and in the Chablais region. Herpetologists conducted random sampling in the three locations and individuals were captured during the reptiles' activity periods, ranging from April to July, i.e. as soon as they came out of hibernation and until temperatures became too high to allow their captures. Specimens were carefully captured by hand.

After data collection and under legal authorization, the specimens were euthanized and then kept frozen at − 20 °C, until transferred to the Museum of Zoology in Lausanne (Lausanne, Switzerland). There, stomach contents were removed and briefly described. According to their size, they were individually stored either in 2 mL Eppendorf tubes or in larger 50 mL falcon tubes in 95 % alcohol at − 80 °C for further analysis.

2.3. DNA extraction and reference sequence database

To increase the yield during DNA extraction, solid parts of the samples were ground with liquid nitrogen (Moorhouse-Gann et al., 2018). To avoid any contamination, mortars and pestles used for grinding were previously immersed in a 2.5 % bleach solution bath for 20 min before washing with ultrapure sterile water (H_2O_{UP}), followed by subsequent washing with 70 % alcohol and UV irradiation. The 2 mL tubes containing liquid or solid parts too small to be ground were heated up to 56 °C in a ThermoMixer® (Eppendorf, Switzerland) until complete alcohol evaporation, before being resuspended in 100–250 μL of H_2O_{UP}.

Genomic DNA extraction was performed on approximately 100 mg of material, with the QIAamp® PowerFecal® DNA kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The concentration and the quality of the extracted DNA were evaluated using a NanoDrop™ 1000 spectrophotometer (Thermo Fischer Scientific, Switzerland) and a Qubit® 3.0 Fluorometer (Thermo Fischer Scientific) (Ducotterd et al., 2021).

A reference DNA sequence database was performed to complete the NCBI database (Nucleotide database of the National Center for Biotechnology Information (Sayers et al., 2022)) by collecting species from the putative diet (based on the literature) of *Hierophis viridiflavus* (Table A.1). DNAs were extracted with the same QIAamp® PowerFecal® DNA kit from mammalian, avian, amphibian and reptile samples. Reference DNAs were then PCR amplified using primers pairs targeting the COI gene. After purification and concentration assay, PCR products of reference species were sequenced at Microsynth (Balgach, Switzerland) and resulting sequencing were registered in the NCBI database.

2.4. Long DNA metabarcoding resolution and de novo assembly

We used the long metabarcoding method developed by (Ducotterd et al., 2021) from which we only retained the combination of primers pairs targeting the mitochondrial-encoded cytochrome oxidase subunit I (COI) gene. Namely, ModRepCOI-F and ModRepCOI-R (Reeves et al., 2018), COI-CO2 and COI-CO4 (Che et al., 2012), ODO-LCO1490d and ODO-HCO2198d (Dijkstra et al., 2014) and mlCOIintF and jgHCO2198 (Leray et al., 2013). All PCRs were performed in a final reaction volume of 25 μL, consisting of 5 μL MyTaq™ Reaction Buffer (Bioline GmbH, Germany), 2.5 μL of primers of interest (0.5 μM final concentration), 2 Units (0.4 μL) of Bioline MyTaq™ DNA Polymerase (Bioline GmbH), 1 μL of DNA (25 ng/μL), filled up at 25 μL final volume with H_2O_{UP}. For all primers

pairs, PCRs were performed in triplicates (Snider et al., 2022) under the same cycling conditions, as follows: an initial denaturation step at 95 °C for 3 min, followed by 37 cycles consisting in a denaturation step at 95 °C for 20 s, an hybridization step at 52 °C for 20 s (54 °C for the primer pair mICOIintF/jgHCO2198), and an extension step at 72 °C for 20 s. All PCRs were terminated by a final extension step at 72 °C for 20 s. All PCR reactions included positive and negative controls and all reaction media were assembled in a sterile environment provided by a DNA/RNA UV-Cleaner chamber (Biosan, Riga, Latvia) (Kuile et al., 2021).

Once the amplification control electrophoresis gels were performed, each sample was respectively pooled by taking 9 μL of triplicate amplified PCR product from each of the 4 different primer pairs (i.e. total final volume for each amplicons pool per sample of 108 μL (3×9x4)). After purification with the Wizard® SV Gel and PCR Clean-Up System (Promega), pools concentrations were assessed using the Qubit® 3.0 Fluorometer and then diluted to a final concentration of 2 ng/μL. Purified pooled PCR products were then fragmented in AFA microtubes (Covaris, USA), into fragments of 290 bp average size, using a S2 focused-ultrasonicator (Covaris), according to (Ducotterd et al., 2020, 2021). Following the manufacturer's protocol, sequencing libraries were created using the TruSeq® Nano DNA HT Library Prep Kit (Illumina Inc., USA). All samples were sequenced in parallel in two separate Illumina MiniSeq runs, using one High Output and one Mid Output flowcells at 2 × 150 bp paired-end reads length.

Computational processing of resulting sequences was carried out according the protocol developed by (Ducotterd et al., 2020, 2021). *De novo* assembly of the reads was performed for each sample through the genome assembly software SPAdes 3.14 using the metagenome assembly option "metaSPAdes" (Nurk et al., 2017). After amplicons' reconstruction, contigs under 150 bp were removed and the remaining contigs files were blasted against the NCBI Nucleotide database (Sayers et al., 2022), completed by our reference sequence database, using the command line tools BLAST+ . The resulting blasts that were not assigned to the eukaryotic domain and had a < 90 % identity were not considered. To improve the accuracy of the taxonomic level of some identified preys, final results of assignments obtained from the BLAST searches against the NCBI Nucleotide database were checked again by manual blasting these sequences in the BOLD database of Barcode of Life DataSystems (Centre for Biodiversity Genomics of the University of Guelph, Canada) (Ratnasingham and Hebert, 2007; see Table A.2).

2.5. Data analysis

Once the prey consumed by individuals were determined, we performed non-parametric tests to study the diet of *Hierophis viridiflavus*. Species' richness was assessed and corresponds to the number of prey per individual according to the presence or absence of small mammals and reptiles in the stomach contents (Stier et al., 2017).

As Jura 1 offered limited sampling and is in proximity of Jura 2, we first checked if the prey were significantly different before regrouping both sampling sites and analyzing the differences between regions (Jura 1 + Jura 2 versus Chablais). To assess whether region of origin and time period might impact the feeding behaviour of *Hierophis viridiflavus*, we compared its diet between locations with a Kruskal Wallis Test. In order to investigate the temporal variation in the diet of *Hierophis viridiflavus*, we used a Chi-Square Test to analyse prey consumption during the post-hibernation period rather than by month, since sample collection was conducted over a restricted period. We chose, based on the species ecology of the species, to define the early post-hibernation period as beginning with the first capture of the season and continuining until May 15, and the late post-hibernation period, corresponding to all captures made after May 15 (Bonnet et al., 2021).

Furthermore, we calculated the β-diversity between populations from different regions and varying periods. Beta diversity measures the community structure through two specific aspects (Anderson et al., 2006). It calculates the variation in species composition in a directional and non-directional ways. First, it calculates the species composition "turnover", which is a directional measure of the variation in species replacement among sampling units along a given gradient (spatial, temporal, or even environmental). Second, it calculates the "nesting", being the non-directional measure of the difference in species richness between communities, i.e. without reference to a certain gradient. The widely used Jaccard index was applied to assess the dissimilarity measure (Legendre and De Cáceres, 2013).

Data analysis was performed using the RStudio v1.4.1717 (RStudio Team, 2020) integrated development environment of R software v4.1.0 (R Core Team, 2021).

3. Results

Metabarcoding sequencing runs yielded a median sequencing depth of 91.16 Mb per sample. Sequencing data of six samples (MZL46432, MZL46434, MZL46438, MZL41166, MZL46527 et MZL46528) were not taken into account as post-sequencing bioinformatics analyses yielded no results (Table A.3).

Based on the literature, species were found as part of the putative diet of *Hierophis viridiflavus* and DNAs from thirty species, locally present in Western Switzerland, have been either collected or directly extracted from specimens. Their respective COI gene partial sequence have been amplified, sequenced, edited and registered in the NCBI Nucleotide Database to complete it (Table A.4). Note that some of them were the first occurrence for a species (for instance *Natrix maura* and *Vipera aspis*) or provided longer sequences than those available in the current database (for instance for *Bufotes viridis* and *Sorex coronatus*).

3.1. Stomach contents' resolution

Metabarcoding of 94 stomach contents of *Hierophis viridiflavus* identified the presence of various organisms. Of these samples analysed, 56 were from Chablais Vaudois and 38 from Jura (16 from Jura 1 and 22 from Jura 2). Some species in the stomach contents

were successfully identified in 86 samples, i.e. 94.6 % of samples, whom 50 from Chablais and 36 from Jura (14 in Jura 1 and 22 in Jura 2). Forty-one different species were recorded for 180 organisms identified, classified as coming from 67 primary prey, 6 secondary prey, 78 parasites (of both snakes and prey), and 29 environmental DNA sequences (organisms present in the environment and unintentionally ingested) (Table A.3). Primary prey represent species voluntarily consumed by the green whip snake, known to be part of their diet. Whereas secondary prey are species consumed by the snake's own prey, although in some cases, classifying organisms into these categories can be more difficult than expected (Gil et Pleguezuelos, 2001; Gil et al., 2020). A large proportion of *Chromadorea*

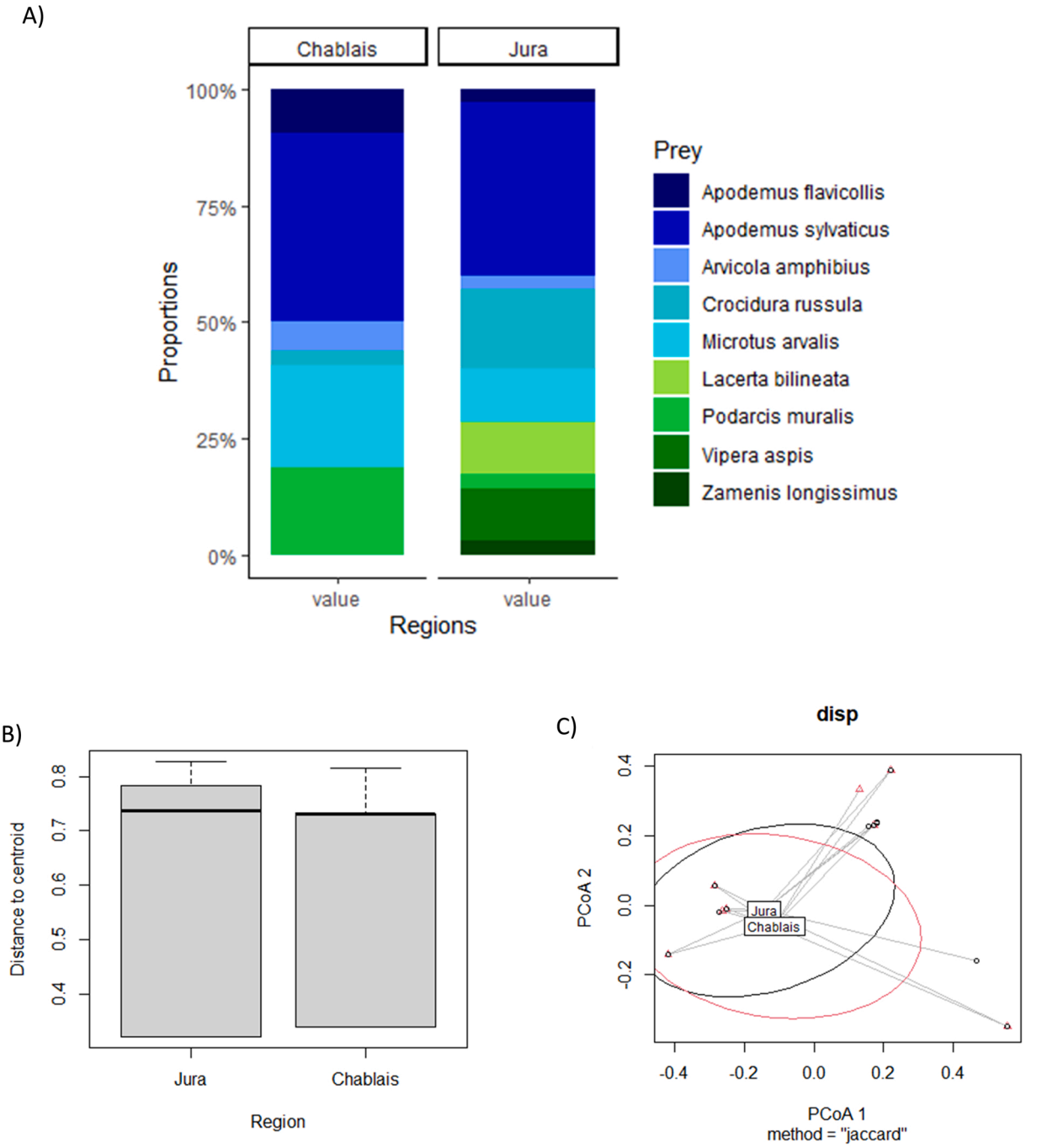

Fig. 1. A) Proportions of prey consumed by introduced Hierophis viridiflavus in the two investigated regions (Chablais and Jura; Western Switzerland). B-C) Boxplot and plot of β-diversity (Jaccard index) in the diet of introduced populations of Hierophis viridiflavus in the two investigated region (Jura and Chablais; Western Switzerland). Black circle and dots correspond to the Jura site and red circle and triangle to the Chablais site.

nematodes were found in these samples. Apart from those and the primary prey, the major diversity of organisms found were Arthropods, mainly insects and arachnids (Table A.5). Additionally, we decided not to use a blocking primer, as *Hierophis viridiflavus* is known to be a cannibalistic species, which could have prevented the identification of such a behaviour. Thus, host DNA was identified in 69.1 % of the samples.

3.2. Diet determination

Among all the species found, some have been determined to be part of the diet. Fifty-five individuals revealed prey consumption (58.5 %). Out of these samples, 27 were from Chablais Vaudois and 28 from Jura (12 from Jura 1 and 16 from Jura 2).

We determined a food spectrum composed of small mammals (76.1 %) and reptiles (23.9 %) with, respectively, 51 and 16 prey items identified. All prey items were identified to the species level with a minimum and maximum identification threshold of 97.9 % and 100 %, respectively. The 55 individuals revealed a total of 67 prey consumed among nine species, classified, as follows, by frequency of occurrence: *Apodemus sylvaticus; Microtus arvalis; Crocidura russula; Podarcis muralis; Apodemus flavicollis; Lacerta bilineata; Vipera aspis; Arvicola amphibius* and *Zamenis longissimus* (see Table A.1). Regarding the richness of the species consumed per individual, most samples contained a single prey (78.2 %), and 12 of them contained two prey items (seven with one micromammal and one reptile and two micromammals in the other five).

3.2.1. Spatial diet survey – regional comparison

Of the 67 prey items found in the stomach contents analysis, 32 were from Chablais Vaudois and 35 from Jura (Jura 1 = 15, Jura 2 = 20). As no differences were detected in the proportion of prey per site (Kruskal Wallis test; p-value = 0.557) and the two Jura sites are geographically close, we decided to investigate *Hierophis viridiflavus*' diet by region, combining the results from Jura 1 and Jura 2.

Our results revealed different proportions of species consumed in the different regions, with a common predominance of *A. sylvaticus*. Only six species were consumed in common between the 2 regions. All 5 species of small mammals and 4 species of reptiles were found in the diet of the green whip snake in the Jura region, whereas, in the Chablais region, this diet is composed of all small mammalian species but only 1 of the 4 species of reptiles, namely *Podarcis muralis* (Fig. 1A; Table A.1). All prey species, excepted *V. aspis* which is not present in the Chablais, were considered and revealed significant differences in their respective proportions by region (Fisher test; p-value = 0.031).

Moreover, although the proportion of species differs among regions, no significant differences were found in the β-diversity of *Hierophis viridiflavus* diet between the Jura and Chablais regions (Anova test; p-value = 0.781). Hence, diet of introduced individuals could be considered homogeneous among the different regions according to the dissimilarity measure (Fig. 1B-C).

3.2.2. Temporal diet survey – time period comparison

The majority of identified prey were consumed by *Hierophis viridiflavus* in April and May as most of the catches were made during these months. No statistical differences in total prey, mammals or reptiles consumption were found between the early post-hibernation period and the late post-hibernation period (Chi-square test; mammals: p-value = 0.480; reptiles: p-value = 0.435).

3.2.3. Vulnerable species' consumption

Among the 9 species consumed by *Hierophis viridiflavus*, three of them are listed in the Swiss Red Lists, namely *Lacerta bilineata*, *Zamenis longissimus, Vipera aspis* and appeared respectively as Vulnerable (VU), Endangered (EN) and Critically Endangered (CR). They represent 33.3 % of the species and 13.4 % of the total prey (Table A.1). At Jura 1 and Jura 2, 20 % of the prey are respectively represented by *V. aspis* and *L. bilineata.*

4. Discussion

Only 55 of the 94 individuals revealed the consumption of one or two prey items. Although stomach material was sometimes found in the remaining individuals, it yielded no usable sequences because the DNA was either insufficient or too degraded (Snider et al., 2022). These results consequently support the idea that a limited number of prey items are present in the stomach simultaneously. This can be explained by a rapid digestion probably induced by a very active metabolism of *Hierophis viridiflavus* (Felicioli et al., 2013). This hypothesis would corroborate with this species' ecology, i.e. thermophilic species with a high body temperature requirement (about 30 °C) that are extremely lively and can move over large distances (racer species) (Lelièvre et al., 2010).

Although extremely accurate, as shown by all species-level assignments, the method used does not provide a quantitative overview of consumed prey. Indeed, amounts of sequence reads present in the samples are not necessarily correlated to species relative abundance and cannot provide information on prey size or stage of digestion (Vasselon et al., 2018). Hence, some prey information cannot be estimated, such as age (adult, juvenile) or sex (if species dimorphism is important). As the majority of stomach contents were in advanced stages of digestion, a morphological examination to compare the metabarcoding results could not be performed. Similarly to other non-invasive methods such as feces analysis of hair, feather or scale, estimating prey gender, life stage or the time elapsed between consumption and sample collection remains very difficult if not impossible (Valentini et al., 2009). The other limitation of the approach used in this study lies in the inability to highlight the cannibalistic feeding behaviour known to occur in *Hierophis viridiflavus* (Capula et al., 2014). If an individual had fed on a snake of its own species, analysis of its stomach contents would have amplified the mitochondrial COI DNA portion of both host and prey, creating an impossibility to distinguish one from the other. This is due to the high genetic conservation of the COI gene with the consequence not to allow the discrimination between individuals of the same

species (McFadden et al., 2011). To avoid this bias, only close observation of feces in order to find scales would be possible (Faraone et al., 2020). Alternatively, the use of highly polymorphic markers, such as specific microsatellites or SNPs, would provide evidences of cannibalism. But, to our knowledge, such methods have never been used in stomach content analyses. This aspect could consequently have an impact of the proportion of reptiles and mammals consumed to an unknown proportion.

A brief comparative study of databases was conducted in this study and supports the utility of considering multiple public repositories of DNA barcode sequences to maximise identification success. In fact, the results of sequence processing obtained on NCBI and then on BOLD showed at times an improvement in the level of identification and determination of the species (Table A.2). It illustrates the need for caution regarding the use of public sequences and, furthermore, raises the question of the counter-productivity of data privatization in science (Meiklejohn, Damaso, et Robertson, 2019).

Among all prey consumed, 76.1 % were represented by mammals. Such a high consumption of mammals might be a result of prey selection, but may also be explained by an important and shared presence of these species on the sampled sites. As for the reptile species, *V. aspis* and *L. bilineata* were frequently consumed where their densities are high and were not found in the Chablais, a site where they are slightly represented (*L. bilineata*) or not at all (*V. aspis*). These results could highlight the opportunistic feeding behaviour of *Hierophis viridiflavus* (Lelièvre et al., 2012b). However, it should be noted that *Hierophis viridiflavus* seems to feed on *Z. longissimus* only occasionally, even when the latter is fairly common (see Chablais site Fig. 1A). The different proportions of Asp vipers consumed depending on the site further support the opportunistic behaviour of the green whip snake (see Table A.1). In Jura 1, a site with a high population density, Asp vipers represented 20 % of the prey. Whereas in Jura 2, where the population is in strong decline, their consumption was estimated at only 5 %. Thus, *Hierophis viridiflavus* diet differs according to the sites and this is partly explained by prey availability. Prey that depend on habitat structure and interactions therein.

According to our results, *Crocidura russula* was mostly consumed in the Jura (Fig. 1A), where large dry meadows with thorny bushes are present and constitute a suitable habitat for this species. On the other hand, *P. muralis* was consumed in greater proportion in Chablais, where a railroad line is present as well as numerous sparsely vegetated areas offering good exposure and refuges for this species. We do not have evaluations of the density of all prey for all populations, also taking into account that the species is quite mobile (and that prey densities are also highly variable). For this reason, we cannot evaluate if some species are predominantly consumed by the green whip snake.

Although we expected to detect more prey later in the season, since individuals are less prone to eat just after being out of hibernation or during the breeding season, diet did not revealed differences between early and late spring. Such a result could suggest that the species emerges rapidly from hibernation and also eats during the mating period (Slip et Shine, 1988). Prey composition also did not show differences between these two periods. However, to detect light differences more data would be required given the numbers of identified prey. Sampling was conducted during the best period to catch this snake, capturing in summer would be much more complicated and would imply a bigger time investment in the field. Moreover, in order to speculate on the hunting activity or differential prey consumption of *Hierophis viridiflavus* over time, it would also be necessary to have representative samples over the years and throughout their period of activity. Even if we have sampled snakes during several years, most of the captures were conducted in 2016 and 2018; but again, the number of prey events and the diversity of them make the analyses of yearly variation complicated. Studying the feeding behaviour of *Hierophis viridiflavus* in function of the seasons and its physiological needs (adult, juvenile; male, female) would be necessary too. This information is all the more essential as *Hierophis viridiflavus* appears to be a quite opportunistic species that might generate different pressures on the herpetofauna depending on these factors. Nevertheless, such a study would required knowledge of all prey population size, while juveniles are very difficult to find and catch (Fornasiero et al., 2017).

In this study, the identification of organisms other than primary prey demonstrates the resolution of the method used, as it revealed snake parasites, as well as prey parasites and other organisms present in the environment (unintentionally ingested). Thus, we determined a substantial part of the species ecology, and not simply its food spectrum. While these findings offer various advantages by simultaneously elucidating trophic interactions, they also present limitations in interpreting the results, as discussed by Tercel et al. in a review of omnivore diet metabarcoding studies (Tercel et al., 2021). Indeed, some of the trophic interactions were revealed, providing insights into the food web and parasitism. Yet, the identification of organisms as secondary prey or environmental DNA remains unclear, especially for some insects found in stomach contents. It cannot be excluded that some of them were voluntarily consumed, for instance samples revealed the presence of invertebrates such as *Noctua comes*, *Amphipyra tragopoginis* and *Horistonotus sp*., without reporting the consumption of insectivorous species (See Table A.3). One of the stomach contents revealed the consumption of the rodent *Apodemus sylvaticus*, as well as the presence of five other invertebrates, namely *Episema glaucina, Lumbricus terrestris, Drilus flavescens, Calliphora vicina and Lampyris noctiluca* (See sample AM69; Table A.3). Under these circumstances and given that the larval stage of the organisms found remains impossible to determine, it becomes difficult to predict which organism was voluntarily consumed, and by whom, and the distinction between primary prey, secondary prey or environmental DNA becomes delicate. Some insight into species ecology, however, may be helpful for this purpose. For example, snakes, due to their limited mouth-gape, are known to be constrained by their own size and their prey's size. Consequently, juveniles are known to consume a higher proportion of insects than adults (Zuffi, 2011). Investigating the proportion of insects consumed between adult and juvenile individuals would be interesting. Such results could provide information on the intention of their consumption, i.e., whether adults rarely consume them, which could suggest inadvertent consumption by eating a large prey item or a deceased prey item carrying insect eggs.

In this study we analysed 94 individuals, 61 of whom reported the presence of one to two previously unreported snake endoparasites in *Hierophis viridiflavus*: in 57 cases nematodes of the *Rhabdiasidae* family and in 15 cases hookworms of the *Ancylostomatidae* family were found. Species of the *Rhabdiasidae* family are spread all over the world and have a wide host range, parasitizing various amphibians, lizards and snakes of Colubridae and Viperidae (Tkach et al., 2014). The limited available sequence data on this family

also explains why the exact species identifications are not reported here. These molecular results suggest a new or still undescribed relationship between *Hierophis viridiflavus* and one parasitic nematodes of the *Rhabdias* genus. As for the hookworm, human intestinal parasites causing helminthic infections are known in *Ancylostomatidae* (Asojo et al., 2005). Nematodes of the *Ancylostomatidae* family are common parasites of vertebrates and particularly of wild mammals; many cases of zoonoses due to cats and dogs infection have been reported (Szwabe et Blaszkowska, 2017). Recently, *Ancylostomatidae* nematode infections have been reported in a freshwater species, *Trachemys venusta*, suggesting a "spill effect" from other cohabiting wild and exotic vertebrate hosts (Flores Peredo et al., 2021). This finding supports the idea that endoparasitic nematode contagion between species is a common phenomenon, as suggested by faecal samples analyses in Wildlife Hospitals in Greece (Liatis et al., 2017). Besides, the invasive turtle *Trachemys scripta elegans*, due to its abundance and adaptability, could be a major vector of parasites and diseases, further threatening the ecosystems where it disseminates (Flores Peredo et al., 2021; Meyer et al., 2015). Thus, the presence of *Ancylostomatidae* endoparasitic nematodes in *Hierophis viridiflavus* may raise several environmental and health issues, as the dispersal of these introduced individuals may lead to the contamination of other native species, including threatened ones, not to mention potential cases of zoonosis (Liatis et al., 2017). The expansion and dispersal of introduced green whip snake populations could be even more damaging than anticipated as they could be a vector of parasites, as is the case between *Trachemys* and the turtles of the genera *Mauremys* and *Emys*, exerting additional pressure on the already vulnerable herpetofauna (Demkowska-Kutrzepa et al., 2018).

Moreover, other cases of parasitism were highlighted in this study. Three prey parasites were discovered, namely *Heligmosomoides polygyrus*, *Hoplopleura sp.* and *Ixodes ricinus*. The gastrointestinal nematode *H. polygyrus* is known throughout Europe to parasitize *Apodemus* species (Zaleśny et al., 2014). In fact, *H. polygyrus* was found in two samples where *A. sylvaticus* was consumed. The lice genus *Hoplopleura*, which was found with *Microtus arvalis*, is a parasitic species that has been previously recorded for such infections in rodents of the Cricetidae family (Cook et Beer, 1955). Finally, *I. ricinus*, whose nymphs are known to parasite mice and lizards (Richter et al., 1991), has been found with *P. muralis* and *A. sylvaticus* as prey. NGS therefore has the capacity to provide information on diets but also on parasites and prey parasites. Indeed, molecular analyses have already revealed parasitic infestation of prey-to-predator in animals (Gakuya et al., 2011). Such information on predator-prey interactions could be very useful for ecosystem conservation and management (Valtonen et al., 2009).

The method developed by (Ducotterd et al., 2020), with a detail observation of the species present in the stomach could also highlight some specific behaviour. Indeed, one of our samples revealed consumption of the European wood mouse, *A. sylvaticus*, simultaneously with a blue fly, *Calliphora vicina*. This necrophagous fly is used in forensic entomology to estimate a post-mortem interval (PMI). *C. vicina* appears with cadaveric odor, i.e. between 36 and 48 h post-mortem (Introna et al., 1998). This unexpected result supports a necrophagic feeding behaviour in *Hierophis viridiflavus*, an event never before recorded in this species. Nevertheless, necrophagy in snakes appears to be a rather occasional phenomenon, although their opportunistic behaviour and the reported cases suggest that this type of event may be more frequent than previously thought (Gomes et al., 2017).

4.1. Does Hierophis viridiflavus impact threatened herpetofauna?

As previously mentioned, in Switzerland, the Aesculapian snake, *Zamenis longissimus*, is classified as Endangered, the Asp viper, *Vipera aspis*, as Critically Endangered and the Western green lizard, *Lacerta bilineata*, as Vulnerable (see Table 1) (Monney et Meyer, 2005). *Hierophis viridiflavus's* diet appears to be composed of 13.4 % of these threatened species, all three sites combined. However, this proportion varies locally. Considering the three introduced populations separately, Jura 1 appears to be the most impacted site with 20 % of Critically Endangered prey, all represented by *V. aspis*. Depending on the site, some threatened species have not or only barely been found. This is due to the fact that they are no longer present or only marginally present. As in the case of the Chablais site, where none of the preys consumed belongs to threatened species. Area where *L. bilineata* and *V. aspis* are now almost absent, which was not the case before the introduction of the green whip snake. These low densities could be explained by the impact of *Hierophis viridiflavus* predation and competition on these populations in the past years. Indeed, in Jura 2, a population of Asp vipers was thriving until a recent strong increase of *Hierophis viridiflavus*, that occurred a few decades after the first introduction (SU, pers. obs.). Subsequently, the green whip snake population expanded in concomitance with Asps' depletion.

Table 1
Species found in the diet of introduced populations of *Hierophis viridiflavus* in Western Switzerland and their Swiss Red List status. LC indicates Least Concern, VU Vulnerable, EN Endangered and CR Critically Endangered. – indicate the lack of the species in the population, 0 the absence of this species as prey. The shades of gray indicate the severity of threat.

Species	Jura 1	Jura 2	Chablais	Total	Swiss Red List Statut
Apodemus sylvaticus	3	10	13	26	LC
Apodemus flavicollis	0	1	3	4	LC
Crocidura russula	3	3	1	7	LC
Arvicola amphibius	1	0	2	3	LC
Microtus arvalis	4	0	7	11	LC
Podarcis muralis	0	1	6	7	LC
Lacerta bilineata	–	4	0	4	VU
Vipera aspis	3	1	–	4	CR
Zamenis longissimus	1	–	0	1	EN
Total	15	20	32	67	

These findings demonstrate the ability of *Hierophis viridiflavus* to prey on threatened species when available. Once again, this attests to its opportunistic feeding behaviour. But their introduction has a significant impact, especially on certain sites with particularly threatened herpetofauna, most notably Asp populations. Finally, it is noteworthy that *Hierophis viridiflavus* induces not only limited predation on the endangered species *Z. longissimus*, but also competition, since these two species have relatively similar diets (Lelièvre et al., 2012b).

5. Conclusion

Through the long DNA metabarcoding based on NGS technology, the diet of *Hierophis viridiflavus* was largely elucidated, and beyond with the description of some elements of the ecosystem trophic interactions, such as parasitism and part of the food web. These results revealed a significant local impact on threatened species depending on the sites and their prey availability, highlighting a highly opportunistic feeding behaviour in *Hierophis viridiflavus*. Moreover, the recurrent presence of endoparasitic nematodes, potentially transmissible to native species, might induce additional pressure on vulnerable species. Our study revealed the need to counter introduced populations' expansion, in order to prevent environmental damages that may sometimes lead to the total extinction of populations of some sensitive species. To further increase the understanding of *Hierophis viridiflavus* feeding behaviour, studying the diet of a larger panel of individuals over a longer period would be necessary. Such knowledge would help to determine which factors influence their feeding behaviour and affect their diet spectrum. This long metabarcoding method could be leveraged in multidisciplinary studies, like in species ecology, whom researches are becoming increasingly challenging with the continuous introduction of non-native individuals.

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Author contribution

AM, JC, FL and SU designed the study, AM conducted 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. AM prepared the original draft, which was then edited and reviewed by all co-authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request. All Raw sequence reads of 96 metabarcodes (94 stomach contents samples from green whip snakes and two negative controls) were registered in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the Bioproject accession PRJNA701542 and the SRA accessions SRR15012261 to SRR15012359. Local reference sequence database consisting to 40 sequences from MW478036 to MW477999 (Table A.3).

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Ethical approval for studies involving animals

Legal authorizations for capture and euthanasia were issued by the Canton of Vaud - Biodiversity and Landscape Division (DGE - BIODIV). Animal samples originate from samples from legal captures of green whip snakes, led in elimination campaigns between 2016 and 2020 under legal authorizations delivered by the State of Canton of Vaud, under the frame of the Federal laws and in compliance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. These samples were then kept in the Museum of Zoology in Lausanne (Lausanne, Switzerland), which kindly tended these samples for being used in this study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2022.e02239.

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