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Short communication

Stable isotopes of a terrestrial amphibian illustrate fertilizer-related nitrogen enrichment of food webs in agricultural habitats

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ABSTRACT

To comprehensively assess the impacts of agricultural practices on biodiversity in complex landscapes mixing both agricultural habitats and remnants of other (presumably more favorable) types of habitats, a prerequisite is to evaluate to which extent agricultural habitats are actually used by a given species. Here, we tested whether the stable isotope method can help to discriminate habitat use of a wild vertebrate, the spined toad (*Bufo spinosus*). We expected habitat to influence their $\delta^{13}C$ values and the use of fertilizers to increase $\delta^{15}N$ values of individuals from agricultural landscapes. Based on 114 toads from seven sites characterized by contrasted habitats (agricultural, forest or mixed habitats), we found that toad blood $\delta^{15}N$ values were positively related to agricultural surface area, a result that was corroborated by diverging blood $\delta^{15}N$ values between habitat categories. Conversely, toad $\delta^{13}C$ values did not vary according to the habitat. Our results suggest that isotopic values (especially $\delta^{15}N$) could be a powerful tool to assess agricultural habitat use in terrestrial taxa. Further studies should usefully investigate whether individual $\delta^{15}N$ values can be used as a fingerprint of other constraints of agricultural habitats (e.g., contaminants) in agricultural landscapes.

1. Introduction

Anthropogenic activities are considered as the main factors responsible for the current loss of biodiversity (Chapin et al., 2000; Myers and Knoll, 2001; Brooks et al., 2002). Among these anthropogenic changes, modern agricultural practices have been shown to negatively influence biodiversity both directly and indirectly. The direct negative impacts of agriculture on flora and fauna are mainly linked to the destruction and simplification of habitat structures (Fahrig, 2003). Indirect effects are mainly mediated by the increasing reliance on chemical inputs that aim at improving crop productivity (Köhler and Triebskorn, 2013). For instance, the use of large amounts of fertilizers can eventually led to disruptions of ecosystem functioning (Huang et al., 2017). In addition, the toxic effects of pesticides on non-target components have attracted considerable interest (Köhler and Triebskorn, 2013). Clearly, both direct and indirect effects are expected to affect the species inhabiting agricultural landscapes (McLaughlin and Mineau, 1995; Köhler and Triebskorn, 2013).

In some cases, it is relatively straightforward to assess the consequences of these habitat modifications on the ecology of animal species (e.g., when nesting trees or shrubs are lacking for birds, Mohring et al., 2021). Yet, in most cases, agricultural landscapes will be intersected with remnants of other types of habitats (e.g., small woods, hedgerows) that should allow the persistence of populations. In these cases, assessing the actual use of agricultural habitats versus remnant of native habitats is logistically complicated, especially when the species under focus is relatively mobile (i.e., most animal species). Nonetheless, in order to comprehensively assess the consequences of agricultural practices on biodiversity, it is necessary to evaluate to which extent such habitat is actually used by a given species (Street et al., 2016).

Stable isotopes can provide insights in this respect (Robinson, 2001; Rubenstein and Hobson, 2004; Perkins et al., 2014; Newton, 2016). The concept of the isotopic niche is based on the fact that an animal's chemical composition is influenced by what it consumes (Fry, 2006). Stable nitrogen isotope values ($\delta^{15}N$) are mostly used as a proxy of trophic position, but can be also a relevant proxy of consumers' foraging

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Table 1
Summary of the sampling design (habitat categories, number of individuals) and of the corresponding toad blood mean (±standard deviation) of $\delta^{15}N$ and $\delta^{13}C$ values.

Sites	Categories	n	Agricultural surface area (ha)	$\delta^{15}N$ (mean ± sd)	$\delta^{13}C$ (mean ± sd)
1	Forest	16	0	0.49 ± 0.54	–24.79 ± 0.26
2	Forest	21	15	1.33 ± 1.61	–23.93 ± 0.53
3	Mixed	16	54	2.52 ± 1.83	–23.62 ± 0.38
4	Mixed	20	104	2.72 ± 1.70	–24.41 ± 0.59
5	Agricultural	19	260	3.43 ± 1.64	–24.35 ± 0.47
6	Agricultural	3	261	7.28 ± 0.20	–23.10 ± 0.76
7	Agricultural	19	286	4.77 ± 1.86	–24.45 ± 0.63

habitat (Kelly, 2000). In our context, because fertilizers widely used in agriculture show relatively high $\delta^{15}N$ (e.g., manure and compost [$\delta^{15}N$ up to 16.2‰], ammonium sulphate [$\delta^{15}N$ up to 6.6‰] and ammonium nitrate [$\delta^{15}N$ up to 2.2‰], Bateman and Kelly, 2007), it is expected that trophic chains influenced by fertilization will be enriched in $\delta^{15}N$ (Anderson and Cabana, 2005; Bateman and Kelly, 2007). As a consequence, individuals relying on agricultural areas to forage should display higher $\delta^{15}N$ values than individuals using other types of habitats. In contrast, stable carbon values ($\delta^{13}C$) vary little along the food chain, and often depend on foraging habitats ($\delta^{13}C$ source). Typically, $\delta^{13}C$ varies among specific primary producers (Farquhar et al., 1989), and we can use this parameter to examine differences in trophic support and thus presumably habitats.

In this study, we tested whether blood $\delta^{15}N$ and $\delta^{13}C$ values can help to discriminate the foraging habitats of a wild vertebrate, the spined toad (*Bufo spinosus*). This species is particularly well suited to test these hypotheses for several reasons. First, this widespread amphibian can live in a variety of habitats and persist even in highly modified agricultural areas (Guillot et al., 2016, see also Salazar et al., 2016; Leeb et al., 2020). Second, spined toads forage for invertebrates and its prey spectrum has been shown to be highly conserved between habitats (Zamora-Camacho and Comas, 2017). Third, the terrestrial part of the life cycle occurs within 1 km from the breeding (sampling) sites, which allow a straightforward classification of the surrounding landscapes potentially used in the day-to-day life of individuals (Janin et al., 2011; Guillot et al., 2016). Finally, the remarkably long lifetime of erythrocytes of amphibians (Altland and Brace, 1962) indicates that stable isotopes from red blood cells actually reflect habitat use during the terrestrial life of our study species prior to breeding (see also Cloyed et al., 2015). In order to test whether isotopic values of spined toads can discriminate their foraging habitats, we assessed $\delta^{15}N$ and $\delta^{13}C$ of red blood cells (herein blood) from 114 individuals from seven sites ranging from forested areas to highly agricultural sites.

2. Material and methods

2.1. Study species

In Western Europe, Spined toad (*Bufo spinosus*) is one of the most common species of amphibians. As most anuran species, spined toads have a biphasic life-cycle with an extensive use of terrestrial habitats during most of the year, and a short breeding season (~1 month) in ponds. During breeding, male toads massively migrate towards ponds where they wait for females (Brischoux and Cheron, 2019) and a large number of males can be easily sampled at each pond.

2.2. Study sites and sampling

Sampling took place in February 2020 on seven breeding ponds situated in the south of the "Département des Deux-Sèvres" nearby the laboratory (46° 8′48.64"N; 0°25'30.86"W). Two sites were located in highly forested areas, three sites in agricultural landscapes, and the remaining two sites at the interface between forested areas and agricultural areas (Appendix 1). Distances between different sites within a habitat type (e.g., ~18 km between the two forested sites; at least ~12 km between agricultural sites) were large enough to minimize spatial autocorrelation (Janin et al., 2011; Guillot et al., 2016). Such site selection allowed making simple habitat classifications (Table 1). Using QGIS.org 2.18.2, 2016 and satellite images (Google Earth), we drew a buffer around each pond (1000 m radius spanning the spatial scale traveled by toads during the breeding migration, Janin et al., 2011; Guillot et al., 2016) and we extracted the surface area of agricultural fields (Table 1).

We focused our sampling on the first breeding males arriving at each breeding sites. Captures occurred at night using a headlamp to locate individuals. Upon sighting, each toad (total N = 114, 3–21 different individuals per sampling sites, Table 1) was captured with a net, and a blood sample was collected (approx. 100 µl) via cardiocentesis using a 1 ml syringe and a 30-G heparinized needle (Brischoux et al., 2018). All individuals were released at their location of capture after blood collection.

2.3. Stable isotope analyses

Whole blood was centrifuged, and red blood cells subsequently stored at −20 °C until analysis. Isotopic analyses were carried out on freeze-dried red blood cells at the LIENSs (La Rochelle, France). Aliquots of ~0.3 mg dry mass were analyzed with a continuous flow mass spectrometer (Thermo Scientific Delta V Advantage) coupled to an elemental analyzer (Thermo Scientific Flash EA 1112). Results are in δ notation relative to Vienna PeeDee Belemnite and atmospheric N_2 for $\delta^{13}C$ and $\delta^{15}N$, respectively. Internal laboratory standards (acetanilide) were used to check accuracy. Measurement errors were <0.15‰ for both $\delta^{13}C$ and $\delta^{15}N$ values.

2.4. Statistical analyses

All data were tested for homogeneity of variance, residues independence and normality with the Bartlett test, Dubin-Watson test and Shapiro-Wilks test, respectively. We also checked the residues normality using diagnostic plots. All statistical analyses were carried out with R. Studio v 1.2.1335 (R Core Team, 2019). We fitted linear mixed models (LMER, package lmerTest, Kuznetsova et al., 2015) to assess differences in blood $\delta^{15}N$ and $\delta^{13}C$ values across agricultural surface area, with "sites" as a random factor. We analyzed these models with variances analysis (ANOVA). We also analyzed differences in blood $\delta^{15}N$ and $\delta^{13}C$ values between categories (see habitat classification). We followed these analyses by post-hoc tests to performed pair-wise comparisons between categories using Tukey-Kramer tests for unbalanced sample sizes (implemented in the TukeyHSD function).

A site was characterized by very low sample size (N = 3, site #6, Table 1, Appendix 1). Excluding this site from our analyses yielded similar results and we present results with the whole dataset below.

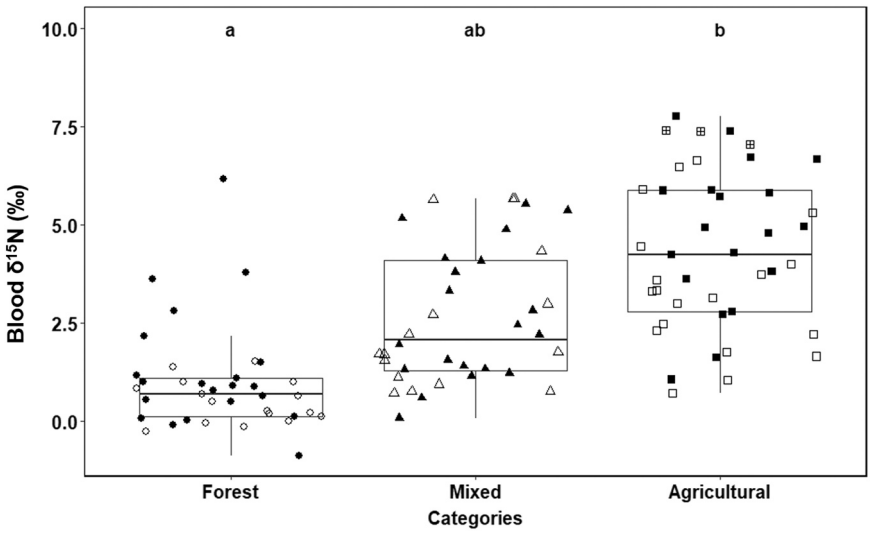

Fig. 1. Blood $\delta^{15}N$ values of toads from forest, mixed and agricultural habitats. The bottom and top of the boxes represent the first and third quartile, the line across the box represents the median, the whiskers represent the minimum and maximum values. The circles, triangles and squares represent individual data points: ○ Site 1, ● Site 2, ▲ Site 3, △ Site 4, ■ Site 5, ⊞ Site 6, □ Site 7. Different letters indicate significant differences.

3. Results

Blood $\delta^{15}N$ values were positively related to agricultural surface area ($F_{1,107} = 16.022$, $p < 0.001$). Blood $\delta^{15}N$ values were significantly different between habitat types ($F_{2,107} = 6.967$, $p < 0.001$, Fig. 1) with post-hoc tests showing that $\delta^{15}N$ of toads from forest habitats were significantly lower than $\delta^{15}N$ of toads from agricultural habitats ($p < 0.001$, Fig. 1), while individuals from mixed habitats were not different from the two other categories (both $p > 0.089$, Fig. 1).

Blood $\delta^{13}C$ values did not vary according to the agricultural surface area ($F_{1,107} = 0.120$, $p = 0.730$). Accordingly, $\delta^{13}C$ values were similar between habitat types ($F_{2,107} = 0.201$, $p = 0.81$).

4. Discussion

Overall, we found that habitat type influences isotopic values of toads. Blood $\delta^{15}N$ values were positively related to agricultural surface area. These results were corroborated by diverging toad $\delta^{15}N$ values between habitat categories with lower $\delta^{15}N$ values found in individuals from forest habitats and higher $\delta^{15}N$ values found in toads from agricultural areas. Conversely, blood $\delta^{13}C$ values of toads did not vary according to the habitat.

Toads that breed in ponds surrounded by agricultural environments, and thus likely living in such environments (Guillot et al., 2016; Salazar et al., 2016; Leeb et al., 2020), were characterized by higher blood $\delta^{15}N$ values than individuals breeding in ponds surrounded by forest. Two different hypotheses could explain such results. First, toads from agricultural landscapes may forage on different food items (higher in the trophic web) than individuals living in forest. This hypothesis seems unlikely as toad diet remains similar amongst habitats (Zamora-Camacho and Comas, 2017). In addition, the remarkably large variation of $\delta^{15}N$ values between habitat types (−0.9 to 7.9‰, Fig. 1), spans approximately 2.1–2.7 theoretical trophic levels (DeNiro and Epstein, 1981). Although proportional abundances of invertebrates of different trophic levels may differ between habitat types (but see Zamora-Camacho and Comas, 2017), it can hardly explain the large variation of $\delta^{15}N$ - hence "trophic levels" - we found. Detailed analyses of the diet of toads and the $\delta^{15}N$ values of prey items in each habitat (see Perkins et al., 2014 for an example of food web stable isotopic table) are required to test this hypothesis. More likely, agricultural fertilization leads to very high amounts of ^{15}N-enriched fertilizers (e.g., manure, compost, ammonium sulphate and to a lesser extent ammonium nitrate, Bateman and Kelly, 2007) deposited on arable land (Tamm, 2012). Relatively high $\delta^{15}N$ values of these fertilizers increases $\delta^{15}N$ baselines that propagate through the trophic webs in agricultural landscapes (Anderson and Cabana, 2005). Such process has already been highlighted in tadpoles developing in waters with high nitrate concentrations (Trakimas et al., 2011).

It is important to emphasize the large variations in $\delta^{15}N$ values between individuals from the same habitat type or study site (Table 1, Fig. 1). Such result seems to suggest different individual strategies of (micro-) habitat use to forage within a similar landscape (Miaud and Sanuy, 2005; Indermaur et al., 2009). This indicates that the influence of nitrogen fertilization on toad $\delta^{15}N$ values is spatially restricted. Such result suggests that, at least in our setting, $\delta^{15}N$ values can help to understand the use of micro-habitats, movement and/or dispersal and individual strategies in agricultural landscapes (Rickers et al., 2006; Dammhahn and Goodman, 2014). Future studies using both individual tracking (i.e, radio-tracking) and isotope analyses will be critical to test for this hypothesis.

In contrast to $\delta^{15}N$ values, our results showed that blood $\delta^{13}C$ values did not differ between toads from agricultural habitats and individuals living in forest. It is likely that the surface area occupied by corn fields (the main C4 plant expected to influence $\delta^{13}C$ values in agricultural habitats, Schwertl et al., 2005) is too low to significantly influence $\delta^{13}C$ values of individual toads using agricultural habitats: In our study area, corn crop represents only ~4.5% of agricultural surface area (Agreste, 2016). Future studies are required to investigate both the presence of toads and their potential prey in different crop types. Additionally the prey of toads could be sampled to assess their trophic level in each habitat to provide a better understanding of the habitat's nutrient flow and its possible influence on toads' use of various habitats (Perkins et al., 2014). Finally, future studies could incorporate other isotope markers such as sulphur or oxygen to further determine the nutrients in each habitat's food web (Penna et al., 2020). Complementary approaches such as compound-specific isotopic analyses of amino acids may well prove useful to assess both fertilizer-related (e.g., phenylalanine) and trophic-related (e.g., glutamic acid) effects on $\delta^{15}N$ (McMahon and McCarthy, 2016).

To conclude, we suggest that $\delta^{15}N$ values could be a powerful tool to assess habitat use in a terrestrial meso-predator such as the spined toad. Whether such approach could be used on other taxa living in agricultural landscape deserve further investigations. Interestingly, $\delta^{15}N$ values, as an index of agricultural habitat use, may also help to reveal individual

susceptibility to disturbances linked to modern agricultural practices (i.e., pesticides). Futures studies should usefully investigate whether individual $\delta^{15}N$ values can be used as a fingerprint of concentrations of contaminants in agricultural landscapes.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2021.107553.

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