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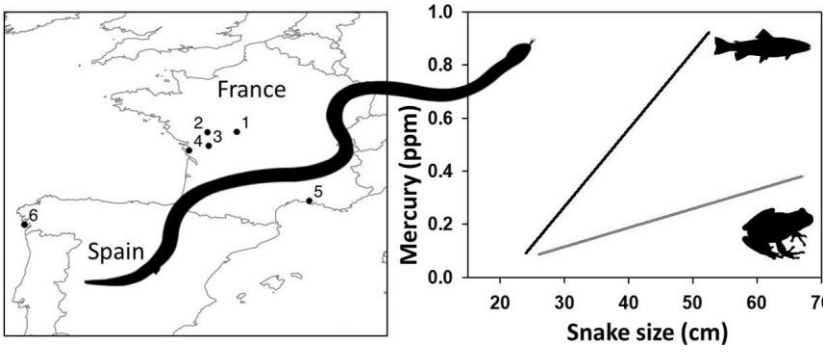
- Hg contamination in freshwater mesopredators has been largely overlooked.
- Hg concentrations were measured in scales of viperine snakes in France and Spain.
- Viperine snakes do accumulate Hg in their scales.
- Diet (fish versus amphibians) influenced accumulation rates of Hg.
- Highest values of Hg were found in individuals from a fish farm.



ABSTRACT

The effects of Hg contamination are presumably widespread across the components of aquatic ecosystems, but investigations have been mainly focused on freshwater fish, because this biota represents a major source of Hg for human populations. Yet, the possible bioaccumulation of Hg on other freshwater meso- and apexpredators (e.g., amphibians, reptiles) has been largely overlooked, especially in Western Europe. In this study, the determinants of Hg concentrations were assessed for the viperine snake (Natrix maura) across 6 populations (>130 individuals sampled in 2016 and 2017) in France and Spain. Specifically, body size, sex, and diet were compared with Hg concentrations measured in ventral scales. Overall, N. maura accumulated Hg in their scales. Sex did not seem to influence Hg concentrations in this species. Significant differences in Hg concentrations were observed between study sites, and these differences were likely to be mediated by site-specific diet. Frog-eating idividuals were characterized not only by lower mean values of Hg (0.194 ± 0.018 μg·g^{-1} versus 0.386 ± 0.032 μg·g^{-1} for piscivorous individuals), but also by weaker slopes of the body size-Hg relationship as compared to fish-eating snakes, suggesting strong differences in accumulation rates due to food resources. Importantly, the highest slope of the body size-Hg relationship and the highest values of Hg were found in individuals foraging on trout raised by a fish farm, suggesting that fish farming may contribute to Hg contamination in inland freshwater systems. Finally, our results are compared with data on Hg concentrations in other species of aquatic snakes, in order to provide a comparative point for future studies.

1. Introduction

Mercury (Hg) is a well-known environmental contaminant which can originate from both natural and anthropogenic sources (Fitzgerald et al., 2007; Selin, 2009). Atmospheric and water circulation patterns tend to concentrate Hg in aquatic environments (Mason et al., 2012). In addition, anoxic conditions found in slow-moving water bodies promote the transformation of inorganic Hg in methyl-Hg by microorganisms (Compeau and Bartha, 1985; Jensen and Jernelöv, 1969), which is known for its toxic effects on humans and wildlife (e.g. Tan et al., 2009; Scheuhammer et al., 2008). Finally, Hg can be bioaccumulated within organisms and

biomagnified through the food chain, and concentrated in apex predators as fishes, birds or mammals (Mason et al., 1995; Atwell et al., 1998; Power et al., 2002).

Over the last decades, the potential deleterious effects of Hg have been investigated from both an ecological and public health perspective (Driscoll et al., 2013; Lavoie et al., 2013; Eley, 1997; Wolfe et al., 1998). Effects of Hg are multiple and cover a large spectrum of syndromes such as neurological dysfunction (Steuerwald et al., 2000; Basu et al., 2005; Clarkson and Magos, 2006; Scheuhammer and Sandheinrich, 2007; Depew et al., 2012), endocrine disorders (Wada et al., 2009; Meyer et al., 2014) or altered reproduction and offspring quality (Klaper et al., 2006; Burgess and Meyer, 2008; Bergeron et al., 2011; Hopkins et al., 2013a; Tartu et al., 2013). Heretofore, the comprehensive assessments of Hg contamination across complex environments and trophic webs are challenging. Indeed, although the toxic effects of Hg are presumably widespread across the components of aquatic ecosystems, investigations of Hg contamination have been mainly focused on freshwater fish (e.g., Depew et al., 2013; Åkerblom et al., 2014; Scheuhammer et al., 2014; Eagles-Smith et al., 2016), probably because this biota represents both a major source of protein for many human populations (Futsaeter and Wilson, 2013; Dong et al., 2015; Lepak et al., 2016; Fliedner et al., 2016). Aquatic birds have also attracted considerable scientific attention in this respect (Ackerman et al., 2016; Jackson et al., 2016; Blukacz-Richards et al., 2017; Sullivan and Kopec, 2018; Żarski et al., 2017; see also Whitney and Cristol, 2017 for a review). Yet, other aquatic vertebrates than fishes, and especially mesoand apex-predators, may well suffer from Hg contamination (e.g. Driscoll et al., 2007). Such overlooked organisms include amphibians (e.g., Bergeron et al., 2010; Todd et al., 2011), aquatic snakes (e.g., Burger et al., 2005; Drewett et al., 2013), and turtles (e.g., Meyer et al., 2014; Slimani et al., 2017). This is especially true in Western Europe where investigations of Hg contamination in aquatic tetrapods are very scarce as compared with other geographic areas such as Northern America. Nevertheless, inclusion of these organisms is of crucial importance if we are to globally assess Hg contamination worldwide, and in turn to monitor its effects on biodiversity and human health (Gustin et al., 2016).

In addition, some lineages of these overlooked meso- and apex predators also provide a unique set of features that make them useful biological tools to monitor Hg contamination in the wild (e.g., Burger et al., 2005; Slimani et al., 2017). For instance, as compared with highly mobile fish and birds, aquatic reptiles and amphibians are characterized by high levels of philopatry associated with relatively low capacities for large scale movements (Hillman et al., 2014). As a consequence, Hg concentrations in their tissues should strongly reveal those of their relatively small home ranges while highly mobile organisms such as fish and birds may provide information that integrates Hg contamination over large distances, and thus, different environments (Burger et al., 2007; Drewett et al., 2013; Slimani et al., 2017). Additionally, aquatic reptiles are situated relatively high in the trophic web, and as ectotherms, they display relatively low metabolic rates and relatively high tissue conversion rates of their food resources which should enhance their capacity to integrate long-term Hg contamination in their tissues. Characterized by an indeterminate growth, many aquatic reptile species display very wide size range between minute neonates and large adult individuals which allow access to bioaccumulation processes within a population. Finally, easily accessible tissues such as claws in turtles or scales in snakes, in which Hg tends to accumulate and bind to keratins (Hopkins et al., 2013b), provide a powerful opportunity to adopt a non-invasive technique in order to assess Hg contamination in these organisms (Schneider et al., 2015).

In this study, Hg concentrations were investigated in a widely distributed European semi-aquatic Natricinae, the viperine snake (Natrix maura). More than 130 individuals distributed across 6 populations situated in France and Spain were sampled. These populations were associated with contrasted ecological contexts and dominant trophic resources (mainly amphibians in 3 sites and fishes in 3 other sites, Table 1). A non-invasive technique (scale-clipping) was used in order to assay Hg concentrations across this wide geographic area. In addition, on a sub-sample of individuals, scale clipping was combined blood sampling to assess the relationship between Hg concentrations in the blood (reflecting short term Hg exposure, i.e., weeks) and in the scales (integrating Hg exposure over a longer time scale, i.e., months) in this species (as shown in other semi-aquatic snake species, Burger et al., 2005). Specifically, the aims of this study were:

1) to assess Hg contamination in this species across a large ecological context,
2) to investigate bioaccumulation rates across a wide range of body size,
3) and to examine the influence of sex and diet on Hg contamination and bioaccumulation rates.

2. Material and methods

2.1. Study species and study sites

The viperine snake (Natrix maura) is a semi-aquatic freshwater natricine widely distributed across Western Europe and Northern Africa, broadly from France to Morocco (Miras et al., 2015). This relatively small-sized species (up to ~80 cm total length) typically forages for fish and amphibians in aquatic environments such as streams, rivers, marshes, and lakes (Miras et al., 2015; Santos and Llorente, 2009). A skin-shedding occurs in N. maura at the onset of the activity period in early spring, while another shedding cycle is later associated with ovulation shortly before oviposition in females (JuneJuly). Our sampling occurred in late spring between these two periods.

The six study sites were distributed in France and Spain and cover the different habitat types in which N. maura typically occurs (Table 1, see also graphical abstract). From north to south, the study sites were 1 - Réserve Naturelle de Chérine within the Brenne Natural Park, France (hereafter "Brenne"), 2 - Lac du Cébron, France (hereafter "Cébron"), 3 - The Boutonne river at Fontenille-Saint

Martin d'Entraigues, France (hereafter "Fontenille"), 4 - The Réserve Naturelle de Moëze-Oléron, France (hereafter "Moëze"), 5 - The Réserve Naturelle de la Tour du Valat, France (hereafter "Tour du Valat"), and 6 - Ons island, Spain (hereafter "Ons").

Table 1
Description ofthe study sites. Site number refers to their location in the graphical abstract. Diet was assessed through opportunistic regurgitations obtain in the field. Identified frog species were Pelophylax spp. for Brenne, Pelophylax spp. and Hyla meridionalis for Moëze and Tour du Valat. Identified fish species were Perca fluviatilis and Gymnocephalus cernua for Cébron, and Blennius spp. for Ons.

Number	Name	Coordinates	Habitat type	N (females/males)	Diet	Sampling period
1	Chérine	46°47′24.18″N, 1°12′2.79″E	Small lakes and ponds	11 (6/5)	Amphibians	May–June 2016
2	Cébron	46°46′5.47″N, 0°11′46.85″W	Artificial lake	22 (14/8)	Fish	May 2016
3	Fontenille	46° 7′26.07″N, 0°8′23.98″W	Fish farm	27(16/11)	Fish	April–June 2016
4	Moëze	45°54′14.16″N, 1°4′22.33″W	Atlantic wetlands and channels	26(13/13)	Amphibians	April–June 2016
5	Tour du Valat	43°30′32.82″N, 4°39′49.14″E	Mediterranean wetlands and	29(17/12)	Amphibians	April–June 2016
6	Ons Island	42°22′32.59″N, 8°56′10.22″W	Oceanic island	13 (3/10)	Fish	June 2017

2.2. Field procedures and sampling

Snakes were captured between April and June 2016 and 2017 (Table 1) by hand either under artificial refuges deployed for snake captures or upon sighting. Snakes were measured on a flexible ruler (snoutvent length, SVL; and total length, TL, f0.5 cm), weighted on a digital scale (f0.1 g), and sexed by eversion of the hemipenis. Snakes were individually marked by ventral scale-clipping followed by heat branding using a surgical cautery (Bovie Medical Corporation). The scale clips obtain after marking were collected, dried on paper towel, and stored at ambient temperature in closed tubes for further Hg analyses (see below).

On a subsample of snakes from Tour du Valat (N = 8), we also collected blood samples in order to assess the relationships between blood and skin (scales) Hg in N. maura. Blood (~150 μl) was obtained through cardiocentesis with a heparinized 30-gauge needle and a 1 ml syringe. Whole blood was stored at −20 °C in sealed tubes until Hg analyses (see below).

At the end of the field procedures, all snakes were released at their place of capture usually within 30 min.

2.3. Mercury assays

Total Hg concentrations in skin and whole blood were determined using an atomic absorption spectrometer AMA-254 (Advanced Mercury Analyser-254; Altec®). At least two aliquots of 1–5 mg of subsamples for each individual were analysed. The accuracy and reproducibility of the Hg measurements was assessed by the analyses of certified reference material (CRM) TORT-2 (Lobster Hepatopancreas from the National Research Council of Canada; certified Hg concentration: 0.27 f 0.06 $\mu g \cdot g^{-1}$ dw) at the beginning and at the end of the analytical cycle, and by running CRM for every 10 samples (Bustamante et al., 2006). Measured values were 0.241 f 0.014 $\mu g \cdot g^{-1}$ dw, n = 17 (recovery 90 f 3%). Mass of the CRM was adjusted to represent an amount of Hg similar to that in the samples. Blanks were analysed at the beginning of each set of samples and the limit of detection was 0.005 $\mu g \cdot g^{-1}$ dry weight (dw). Hg concentrations in snake tissues further are expressed in $\mu g \cdot g^{-1}$ dw.

3. Results

3.1. Scale versus blood Hg concentrations

Hg concentrations between blood and scale clips were positively correlated ($F_{1,6} = 8.87$, $r^2 = 0.59$, $p = 0.02$). However, blood Hg concentrations were 3 times higher than scale Hg concentrations (paired t-test, $t = 8.01$, $p < 0.0001$, 0.719 f 0.267 $\mu g \cdot g^{-1}$ versus 0.237 f 0.195 $\mu g \cdot g^{-1}$, respectively).

3.2. Determinants of Hg levels

Overall, a significant positive relationship was observed between snake size (SVL) and Hg concentration ($F_{1,130} = 43.95$, $r^2 = 0.25$, $p < 0.0001$). Within sites, the same trend was detectable (all $p < 0.005$, Fig. 2), except for Tour du Valat ($p = 0.16$, Fig. 1). The strength of the relationship was highly variable between sites, with three sites characterized by relatively weak slopes (Brenne, 0.009; Moëze, 0.007) and/or non-significant correlation (Tour du Valat), and three sites characterized by steeper slopes (Cébron, 0.019; Fontenille, 0.029; Ons, 0.015, Fig. 1). Interestingly, these two sets of sites differed according to the inferred diet of the snakes ($\chi^2 = 132$, df = 5, $p < 0.0001$, Figs.1 & 2).

Accordingly, differences in Hg concentrations were found between sites (ANCOVA with the SVL as the covariate, $F_{5,124} = 11.81$, $p < 0.0001$, Fig. 2), and also a strong influence of inferred diet on Hg values with fish-eating snakes having higher Hg concentrations than frogeating ones (ANOVA, $F_{1,129} = 30.63$, $p < 0.0001$, 0.386 f 0.032 $\mu g \cdot g^{-1}$ versus 0.194 f 0.018 $\mu g \cdot g^{-1}$ respectively, Fig. 2).

Finally, there were no sex related differences in Hg concentrations, either across all study sites ($F_{1,126} = 0.56$, $p = 0.46$), or when analyses were restricted to sites with balanced samples between males and females, and with $N > 10$ individuals in each category (Tour du Valat $F_{1,26} = 0.08$, $p = 0.78$; Moëze $F_{1,23} = 2.09$, $p = 0.16$; Fontenille $F_{1,25} = 0.18$, $p = 0.68$).

4. Discussion

This study provides an insight into Hg bioaccumulation in a freshwater mesopredator, the viperine snake (N. maura), over a large spatial range and across contrasting habitats. Overall, N. maura do accumulate Hg in their scales. Hg concentrations between blood (short-term Hg exposure, i.e., weeks) and scales (integrating Hg exposure over a longer time scale, i.e., months) were correlated, but that blood display higher Hg concentrations than scale clips. Sex did not seem to influence Hg concentrations in this species. Finally, strong differences of Hg concentrations between study sites that are likely to be mediated by sitespecific diet. Frog-eating individuals were characterized not only by lower mean values of Hg concentrations, but also by weaker slopes of the body size-Hg relationship as compared to fish-eating snakes, suggesting differences in Hg accumulation rates due to food resources. All of these findings are discussed sequentially below.

A positive correlation was observed between Hg concentrations quantified in skin (scale clips) and blood. As already demonstrated in other studies, this suggests that scale clipping (a non-invasive sampling technique) is a valuable method for monitoring Hg contamination in snake species (Burger et al., 2005) and that N. maura can be used as a bio indicator of Hg contamination. Interestingly, despite this broad relationship, significant differences were found in Hg values, with blood showing Hg concentrations three times higher than skin (0.719 f 0.267 $\mu g \cdot g^{-1}$ versus 0.237 f 0.195 $\mu g \cdot g^{-1}$). Because these two tissues integrate Hg exposure at different time-scales (weeks for blood versus months for skin), this result suggests a possible seasonal shift in the feeding ecology of snakes from the Tour du Valat population, or less likely a seasonal shift in Hg content of their prey. It is likely that foraging ecology of this species, and especially prey availability and catchability shifts during the spring. Indeed, amphibians (with presumably lower Hg concentrations, see below) are more abundant in early spring (e.g., amphibian breeding season and activity peak), while we can posit that fish (with presumably higher Hg content, see below) become progressively more important in the diet of N. maura with increasing water temperatures that may allow better rates of fish capture

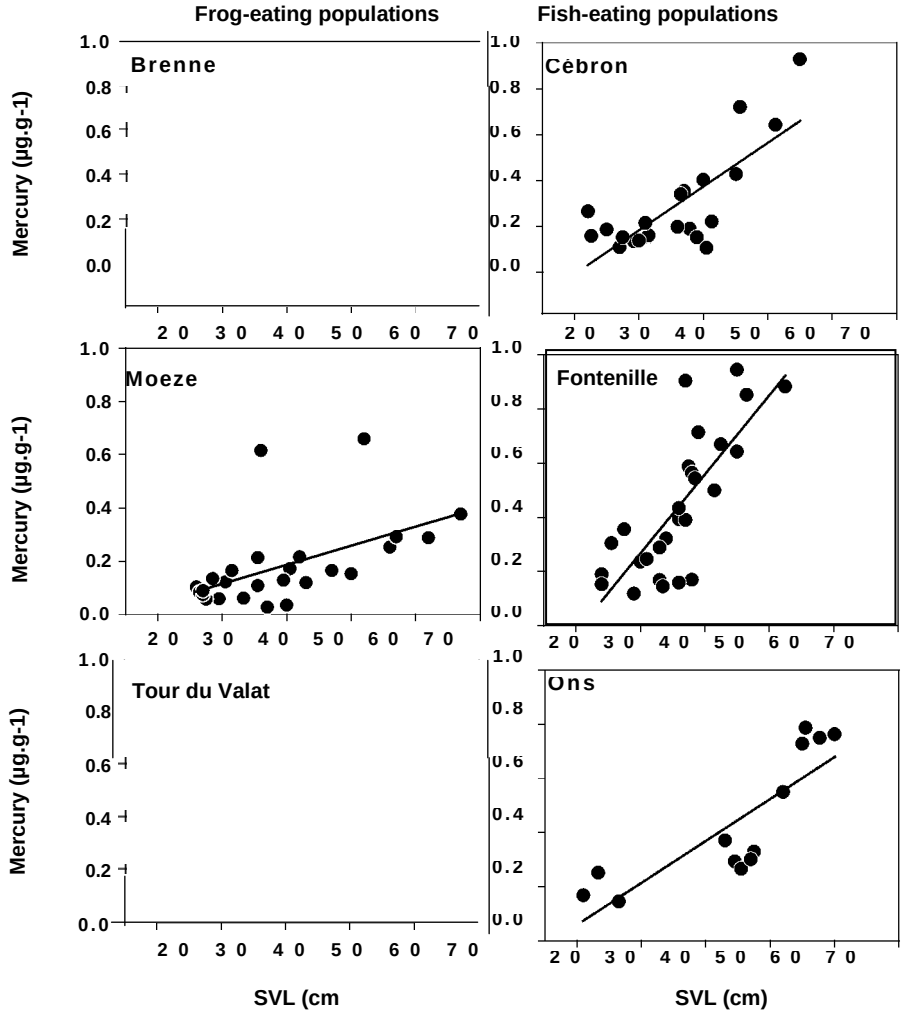


Figure 1 : Relationship between body size (snout-vent length) and Hg concentration measured in scale-clips. Left-hand side panels show data for frog-eating populations while right-hand side panels show data for fish-eating snakes.

Interestingly, the relatively high degree of variation of Hg concentrations between skin and blood in N. maura seem relatively uncommon in other species that share similar ecology (semi-aquatic snake species), where skin and blood Hg values tend to be very similar (Table 2). Importantly, the seasonal variation in Hg exposure suspected in N. maura from the Tour du Valat population may induce intense seasonal pulses in Hg contamination in this species, and the ecotoxicological consequences of these seasonal pulses (as compared to an exposure to constant values of Hg) clearly deserve further attention.

Although sex differences in Hg concentrations are expected if females transfer maternal Hg to their eggs during vitellogenesis (Hopkins et al., 2004), there was no influence of sex on Hg concentrations either across or within our study populations. Some of our sample sizes were modest, and may have hampered our ability to detect such difference. However, this was not the case for all of our study sites (see Table 1). Alternatively, we can hypothesise that the foraging ecology of males and females did not differ in our study populations, especially as body size were very similar between sexes in our sampled individuals. This result may also reflect the time period of the study. While Hg concentrations in the skin integrates Hg contamination occurring since the last shedding cycle, maternal transfer of Hg in eggs en-compasses the whole vitellogenesis process (i.e., from early spring to early summer when oviposition occurs).

Table 2

Review of Hg concentrations assessed in freshwater semi-aquatic snake species in Western Europe (this study) and NorthernAmerica. SVL stands for snout-vent length. Hg levels are given as the mean f SE (µg.g^{-1} dw).

Species	Site	Tissue	SVL (cm)	Hg (µg·g^{-1})	Reference
Natrix maura	Brenne, France	Skin	32.3 f 12.0	0.145 f 0.071	This study
	Cébron, France	Skin	35.6 f 8.8	0.289 f 0.218	
	Fontenille, France	Skin	35.9 f 6.9	0.439 f 0.261	
	Moëze, France	Skin	39.5 f 11.7	0.183 f 0.159	
	Tour du Valat, France	Skin	38.6 f 8.1	0.220 f 0.153	
		Blood	39.2 f 6.4	0.719 f 0.267	
	Ons, Spain	Skin	44.5 f 13.1	0.438 f 0.241	
Agkistrodon piscivorus	Savannah River Site, South Carolina, USA	Blood	57	0.1 f 0.04	Burger et al., 2006
Nerodia fasciata	Savannah River Site, South Carolina, USA	Blood	51	0.400 f 0.047	Burger et al., 2006
Nerodia sipedon	East Fork Poplar Creek, Tennessee, USA	Skin	–	0.423 f 0.028	Burger et al., 2005
		Blood	–	0.407 f 0.042	Burger et al., 2005
	East Fork Poplar Creek, Tennessee, USA	Skin	61	0.500 f 0.045	Campbell et al., 2005
		Blood		0.372 f 0.046	Campbell et al., 2005
	Little River, Tennessee, USA	Skin	57.5	0.365 f 0.032	Campbell et al., 2005
		Blood		0.436 f 0.065	Campbell et al., 2005
	Raritan Canal, New Jersey, USA	Skin	~55	0.159 f 0.023	Burger et al., 2007
		Blood		0.128 f 0.026	Burger et al., 2007
	South River in Waynesboro, Virginia, USA	Blood	59	2.24 f 0.42	Drewett et al., 2013
Nerodia taxispilota	Savannah River Site, South Carolina, USA	Blood	59	0.7 f 0.015	Burger et al., 2006

Therefore, if maternal transfer of Hg occurs in this species, we can expect that Hg concentrations in females would decrease during vitellogenesis, and that such diminution would be detectable after the second skin shedding cycle (early summer). Future studies would usefully explore temporal variation of Hg concentrations in the scale-clips of both males and females, in order to assess putative maternal transfer of Hg to the eggs, and to assess sex differences in Hg contamination rates.

The Hg concentrations quantified in N. maura are comparable to those recorded in other species sharing a similar ecology (semi-aquatic snake species feeding on both fish and amphibians, Table 2). Yet, in our study, N. maura was characterized by an overall smaller body size than the other species (Table 2), which suggest that accumulation rates may be higher in this species. Additionally, a strong influence of the study site on Hg concentrations in N. maura was found that seemed to be mediated by site-specific diet. Indeed, sites where N. maura consume predominantly amphibians were characterized not only by lower mean values of Hg, but also by relatively weaker slopes of the body size-Hg relationships, suggesting strong differences in accumulation rates between sites. Because amphibians (frogs) are situated at lower trophic levels than fishes (Vander Zanden et al., 1997), and because they rely mostly on airborne and terrestrial food sources (insects), they may dis-play both lower Hg concentrations and higher proportion of inorganic Hg that fish that feed on aquatic food sources. Such probable difference in Hg concentrations of fish versus frogs likely explains the strong influence of diet on Hg values, with relatively lower concentrations in frogeating as compared to fish-eating snakes. Yet, the possible influence of diet on Hg concentrations in N. maura we hypothesised will necessitate specific investigations in order to assess Hg values in prey (frogs versus fish) in our different study sites, as well as assessing the relationships between trophic levels of both the snakes and their prey (i.e., measured through stable isotopes) and Hg contamination.

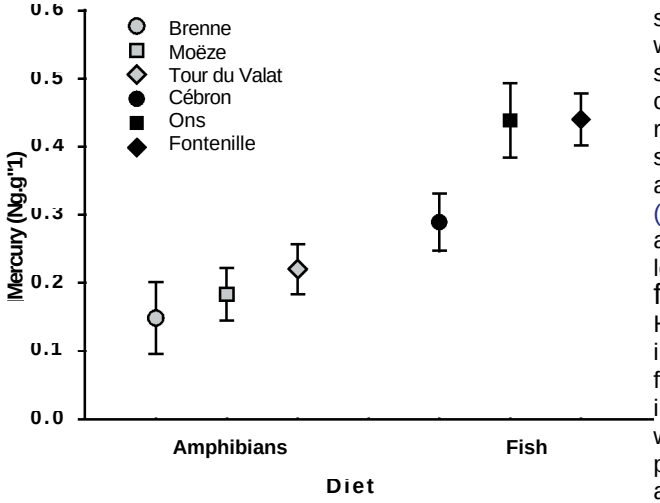


Figure 2 : Mean Hg concentrations (±SE) measured in scale-clips. Grey symbols represent frog-eating populations, while black symbols show fish-eating snakes. For clarity, data are presented by ascending order

Finally, we emphasize the specific case of the Fontenille population of N. maura, which display both the highest slope of the body size-Hg concentration relationship and the highest value of size-specific Hg values with value reaching almost 1 $\mu g \cdot g^{-1}$ in individuals <45 cm SVL, as compared to similar values in snakes >55 cm SVL in other populations (Figs.1 and 2, see also Table 2). This study site is situated on a fish farm, and N. maura from this population feed heavily on the fry and juveniles trouts raised by the fish farm. The relatively higher value of Hg concentrations and Hg accumulation rates in this population dovetail remarkably well with processes that have recently been described in highaltitude aquatic ecosystems (Hansson et al., 2017). In this study system, farmed trout raised on commercial pellets based on proteins from fishery products of marine origin show both high Hg concentrations and Hg isotopic signatures that are comparable to that of top-predator marine biota (Hansson et al., 2017). It is likely that the commercial pellets used to raise trouts at the Fontenille fish farm are also based on proteins from fishery products of marine origin, and that introduction of marine Hg through the fish food is responsible for the higher value of Hg concentrations and Hg accumulation rates in this N. maura population. Interestingly, both Hg concentrations and Hg accumulation rate of the Fontenille population are even higher than the Ons population which feed on marine fish (Galân, 2012). Future studies should usefully assess the relative contribution of fish farming on Hg contamination in aquatic ecosystems and its role as a vector of Hg of marine origin in inland freshwater systems (Hansson et al., 2017).

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