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Cover photograph: American bullfrog (*Lithobates catesbeianus*) from Griesbroek, Balen; see paper by DESCAMPS & DE VOCHT (photograph by Sarah Descamps).

University of Antwerp

Dear All,

We are very happy to announce the upcoming **Zoology2016 congress** and its **CRC-satellite symposium,** taking place from 15 to 17 December 2016 at Antwerp University and the Antwerp ZOO. This year's edition will be the 23th zoology congress organised annually on behalf of the Royal Belgian and Dutch Zoological Societies. At the Zoology Congress, zoologists studying life in all its aspects (from molecules to ecosystems) will meet and discuss. Young scientists are particularly invited to participate, but all zoologists, at any stage of their careers, are very welcome to submit.

The theme for the keynote presentations is **Nature conservation in a changing world**. Our planet is in the midst of a wave of man-made extinction. Reversing this dreadful trend will require input from various fields in biology and beyond. In an attempt to stimulate a multidisciplinary approach to conservation biology, Zoology 2016 has invited four keynote speakers who are studying zoological biodiversity issues from different angles. *However, the organizers await submissions from all fields in animal sciences.*

Linked to the keynote theme, the Antwerp ZOO Centre for Research and Conservation (CRC) will present the results of its research projects in zoo-science and conservation to a general public during the satellite symposium.

Please, visit the website at www.zoology2016.be for more detailed information, registration and abstract submission.

We look forward to meeting you at Zoology 2016.

Prof. Gudrun De Boeck, (Chair Scientific Committee)
Prof. Peter Aerts, (Chair Organising Committee)

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keynote presentations on:

16-17 December **Nature conservation in a changing world**

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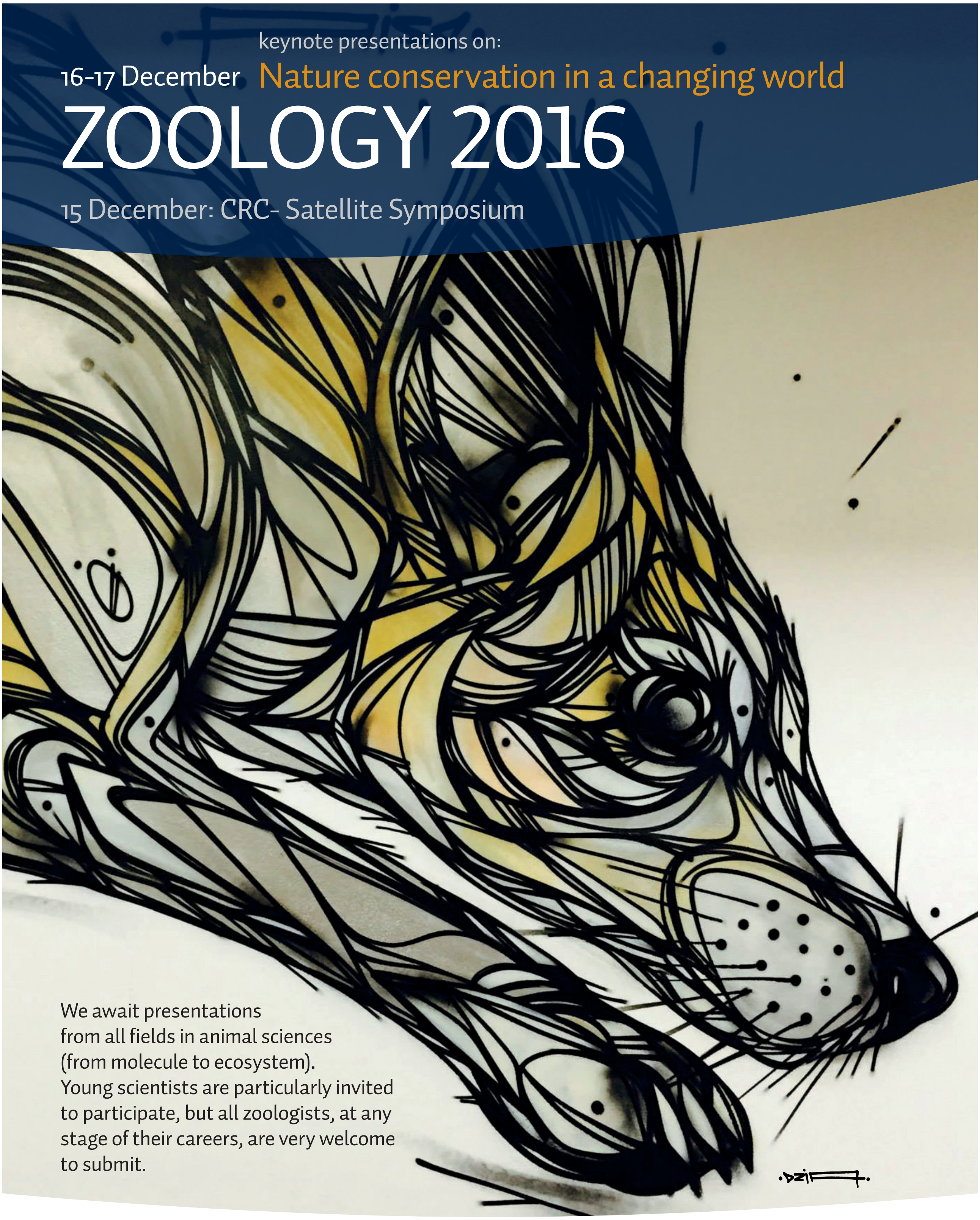
15 December: CRC- Satellite Symposium



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Changes in prey importance and prey niche overlap of sexes during the alpine newt breeding season

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ABSTRACT. Urodeles, including European newts, are usually sexually dimorphic predators. Among newts, the alpine newt has the most pronounced sexual size dimorphism (in favour of females). Gender is a factor that is often associated with intra-specific diet differences. Despite the significant number of dietary studies on the alpine newt, some topics such as the breadth of the trophic niche and its overlap between sexes, or inter-sexual differences in qualitative and quantitative composition of prey remain unresolved. The present study dealing with these questions was conducted at two localities (ponds at an elevation of about 450 m) in the Czech Republic. Newts were captured from the banks during the entire breeding season using a dip net, and the stomach contents were extracted using a stomach flushing technique. Altogether 190 individuals were sampled, and a total of 1,417 prey items were obtained. The available food sources differed over the course of the breeding season, as newts changed the taxa they preyed on. This reflects the ability of newts to switch between several hunting strategies. The overall food niche overlap between the sexes was relatively large (C = 0.761, resp. C = 0.797). Inter-sexual differences were detected at both localities, mainly in the number of prey items consumed from the most important prey categories such as *Rana* eggs or Isopoda, which were consumed in higher numbers by females. The findings of this study suggest that females are more sensitive to the trade-off between energy intake and expenditure during the breeding season.

KEY WORDS: Amphibia, Caudata, food, foraging, diet

INTRODUCTION

For many predators, including amphibians, our understanding of what is eaten, how the diet is influenced by foraging behaviour, and the consequences of the diet and foraging in relation to body condition is limited. This is partly due to the fact that predator diets are influenced by individual factors such as age, size or sex, and simultaneously by factors of their environment such as prey size, diversity and abundance, the last two of which are often difficult to measure (Beck et al., 2007).

Sexual size dimorphism is an intrinsic factor that is often associated with intra-specific diet differences (Shetty & Shine, 2002; Blackenhorn, 2005; Zalewski, 2007). In turn, inter-sexual resource partitioning can itself directly lead to sexual size dimorphism (Shine, 1989; Krüger, 2005).

Sexual size dimorphism has also been reported for European newts, and among them the one with the most pronounced sexual size dimorphism is the alpine newt (*Ichthyosaura alpestris*) where females are significantly larger than males (Denoël et al., 2009). The alpine newts have a multiphasic life cycle – seasonally changing between aquatic and terrestrial environments. During their aquatic phase they reproduce (Griffiths, 1996). Newts concentrate in lentic waters at higher densities than they do in the terrestrial environment, and catching them in water is easier (Hoeck & Garner, 2007). Therefore, most studies of newt ecology, including feeding ecology, are realized during their breeding period. When living syntopically

Alpine newts stay in the water for a shorter time than other species (COVACIU-MARCOV et al., 2010).

Newts are able to hunt prey just as effectively on land as in the water (HEISS et al., 2013). The availability and consequent importance of prey items is specific according to the locality (e.g. RULÍK, 1993; DENOËL & ANDREONE, 2003). Densities and composition of prey are not stable over the season and can vary significantly over the course of the breeding period (SCHABETSBERGER & JERSABEK, 1995; DENOËL & DEMARS, 2008). It is therefore important to examine the entire breeding period to obtain a complete picture of newt prey composition.

In previous studies on the diet of alpine newts in the Czech Republic, we dealt with non-prey items (KOPECKÝ et al., 2011), scaling of prey between the sexes during the breeding season (KOPECKÝ et al., 2012a) and with the composition of the diet during spring migration (KOPECKÝ, accepted). The present study uses the same dataset as previously published studies, and by highlighting two so-far neglected topics - prey change and dietary overlap of the sexes - completes a picture of the alpine newt's diet. Therefore aims of this paper are to i) analyse the breadth of the trophic niche and its overlap between the sexes; ii) determine inter-sexual differences in amount of consumed prey from various taxa; and iii) detect changes of consumed prey over time.

MATERIAL AND METHODS

The study was conducted at two localities in the Czech Republic near the town of Ledeč nad Sázavou. Both sites lie at an elevation of about 450 m. The first, hereafter referred to as locality A (49°42'45" N, 15°16'50" E), is a fishless pond with a surface area of 36.0 m² and a maximum depth of 0.8 m. The pond's banks are planted with willows (*Salix* sp.), and the pond is surrounded by a pasture. The bottom is muddy, and vegetation in the water is scant, consisting mainly of common duckweed (*Lemna minor*), startwort (*Callitriche verna*) and compact rush (*Juncus conglomeratus*). The pond is artificial, its original purpose being to drain water from surrounding pastures. The water's pH values ranged from 6.5 to 7.0 during the study.

The second locality, designated as locality B (49°44'24" N, 15°16'59" E), is a now-fishless pond that was historically used for fish rearing. Pond B is situated in a spruce forest. It has a surface area of 27.5 m² and maximum depth of 0.3 m. The pond's bottom is muddy. Water plants, consisting mainly of common waterweed (*Elodae canadensis*), startwort (*Callitriche verna*) and reed mannagrass (*Glyceria aquatica*), cover a large part of the pond's surface area. During the study, pH values were around 5.5.

Alpine newts are the dominant amphibian species at both localities, where a small number of smooth newts (*Lissotriton vulgaris*) and common frogs (*Rana temporaria*) also regularly reproduce. Adult alpine newts were caught during the entire breeding season from the banks using a dip net. Sampling was done during daylight hours: once in April, once in May and once in June 1997. The newts were kept in a container filled with water from the pond and marked (HEYER et al., 1994) by toe-clipping (FERNER, 1979). To avoid data dependence (LUISELLI et al., 2007), only the newts captured for the first time were used in the study. Stomach contents were extracted using the stomach flushing technique described by OPATRNÝ (1980). The newts were not anaesthetized and were released back into the water immediately after these procedures.

The stomach contents were individually stored in vials and preserved in 4% formaldehyde. Prey items were identified using a stereo microscope. Due to the technical impossibility of weighing all the prey items obtained (1417), only representative samples of each taxa (cca 15% from all prey items) were weighed. Obtained weights were verified by previously published work (OPATRNÝ, 1968). Based on this approach four biomass categories were defined: 0.001 g for Megaloptera-larvae, Cladocera, Cyclopoida, Turbellaria; 0.01 g for Chironomidae-larvae, Chironomidae-pupae, Culicidae-pupae, Bivalvia, Gastropoda, Dytiscidae-larvae; 0.1 g for Ephemeroptera-larvae, Ephemeroptera-pupae, Lepidoptera-larvae, Plecoptera-larvae, Trichoptera-larvae, Arachnida, Isopoda, *Rana*-eggs, and 1 g for *Lumbricus*.

The index of relative importance (IRI) and IRI% for each taxon (i) were computed following MARIANO-JELICICH et al. (2007) as:

$$IRI_i = f_i\% \, (n_i\% + m_i\%)$$

$$IRI_i\% = (IRI_i \, . \, 100) \, / \, IRI_{total},$$

where f% is the percentage of newts containing a particular taxon ($_i$), n% is the percentage of prey items of a particular taxon ($_i$) out of all prey items, and m% is the percentage of biomass provided by a particular taxon ($_i$) out of the estimated total biomass consumed.

Trophic niche overlap using Schoener index (KREBS, 1998) was calculated as:

$$C = 1 - 0.5 \, (\Sigma_i \mid p_{xi} - p_{yi} \mid),$$

where p_{xi} is the proportion of taxon ($_i$) in sex ($_x$) and p_{yi} is the proportion of taxon ($_i$) in sex ($_y$).

Food niche breadth was calculated by Levins' index (KREBS, 1998) as:

$$B_A = (B - 1) \, / \, (n - 1),$$

where B_A is the standardized Levins' index by the number of available items n:

$$B = 1 \, / \, \Sigma \, p_i^2,$$

where p_i is proportion of taxon in overall diet ($_i$).

Mann-Whitney U–tests were used for comparing number of consumed prey items between sexes. Calculations were performed in Statitistica 12 software (Statsoft 2012). Statistical significance was determined at the level $\alpha = 0.05$.

RESULTS

During the three study months, a total of 190 newts were sampled (locality A: 47 males, 50 females; locality B: 54 males, 39 females), from which a total of 1,417 prey items were obtained. Based on pooled sample data (sexes and months together) the most consumed prey based on IRI% were Cladocera at locality A and Isopoda at locality B. The importance of food resources was not stable during the breeding season, and shifts among prey categories were considerable (Fig. 1).

The total number of prey items eaten throughout the season from a particular taxon was, with some exceptions, the same for males and females at locality A (Table 1), just as it was at locality B (Table 2). If sampling time is considered, there was no inter-sexual difference in the consumption of any prey category in April, May or in June at locality B. At locality A, females consumed more *Rana*-eggs during April (males: mean = 0.31, min.– max. = 0–3; females: mean = 1.10, min.–max. = 0–4; U-test: Z = 2.13, $P < 0.05$). During June, females consumed more Gastropods (males: mean = 0; females: mean = 0.35, min.–max. = 0–2; U-test: Z= -2.09, $P < 0.05$) and Cladocera (males: mean = 9.75, min.–max. = 0–45; females: mean = 38.5, min.–max. = 0–114; U-test: Z= -2.68, $P < 0.01$).

The overall food niche overlap of sexes (based on n%) was quite large at locality A (C = 0.761) as well as at locality B (C = 0.797). Niche overlap was relatively stable throughout the breeding season at locality B (April, C = 0.895; May, C = 0.833; June, C = 0.868), while at locality A there was an apparent increase in overlap in June (April, C = 0.624; May, C = 0.678; June, C = 0.896).

Levin's niche breadth changed during the breeding season. At locality A niche breadth was widest at the start of the season (in April) for both sexes, whereas at locality B the opposite was true and breadth was widest in June (Table 3).

DISCUSSION

Breeding alpine newts prey on a wide variety of organisms. Generally, Chironomidae larvae (ROČEK et al., 2003; DENOËL & ANDREONE, 2003), Cladocera (JOLY & GIACOMA, 1992) and

TABLE 1

Differences in the number of consumed prey items between sexes of alpine newt from locality A (data from the months April, May, June pooled).

Prey	M mean (min. -max.)	F mean (min. -max.)	Z	P
Turbellaria	0.13 (0-1)	0.26 (0-1)	1.63	0.10
Oligochaeta	0.06 (0-1)	0.18 (0-1)	-1.72	0.09
Gastropoda	0.02 (0-1)	0.16 (0-2)	-1.88	0.07
Bivalvia	0.09 (0-3)	0.04 (0-1)	0.07	0.94
Arachnida	0.00	0.00		
Cladocera	3.32 (0-45)	15.40 (0-114)	-1.73	0.08
Cyclopoida	**0.17 (0-1)**	**0.06 (0-2)**	**2.03**	**0.04**
Isopoda	0.09 (0-1)	0.08 (0-1)	0.08	0.93
Ephemeroptera larvae	0.09 (0-3)	0.16 (0-2)	-1.34	0.18
Ephemeroptera pupae	0.00	0.12 (0-5)	-1.36	0.17
Plecoptera nymfa	0.23 (0-3)	0.22 (0-3)	0.35	0.73
Megaloptera larvae	0.04 (0-1)	0.02 (0-1)	0.63	0.53
Trichoptera	0.06 (0-1)	0.04 (0-1)	0.52	0.60
Lepidoptera	0.06 (0-2)	0.00	1.45	0.15
Culicidae pupae	0.02 (0-1)	0.00	1.01	0.31
Chironomidae larvae	0.66 (0-7)	0.48 (0-6)	0.86	0.39
Chironomidae pupae	0.17 (0-2)	0.48 (0-5)	-0.82	0.42
Coleoptera	0.02 (0-1)	0.02 (0-1)	0.03	0.98
Rana eggs	**0.17 (0-3)**	**0.58 (0-7)**	**-2.24**	**0.03**

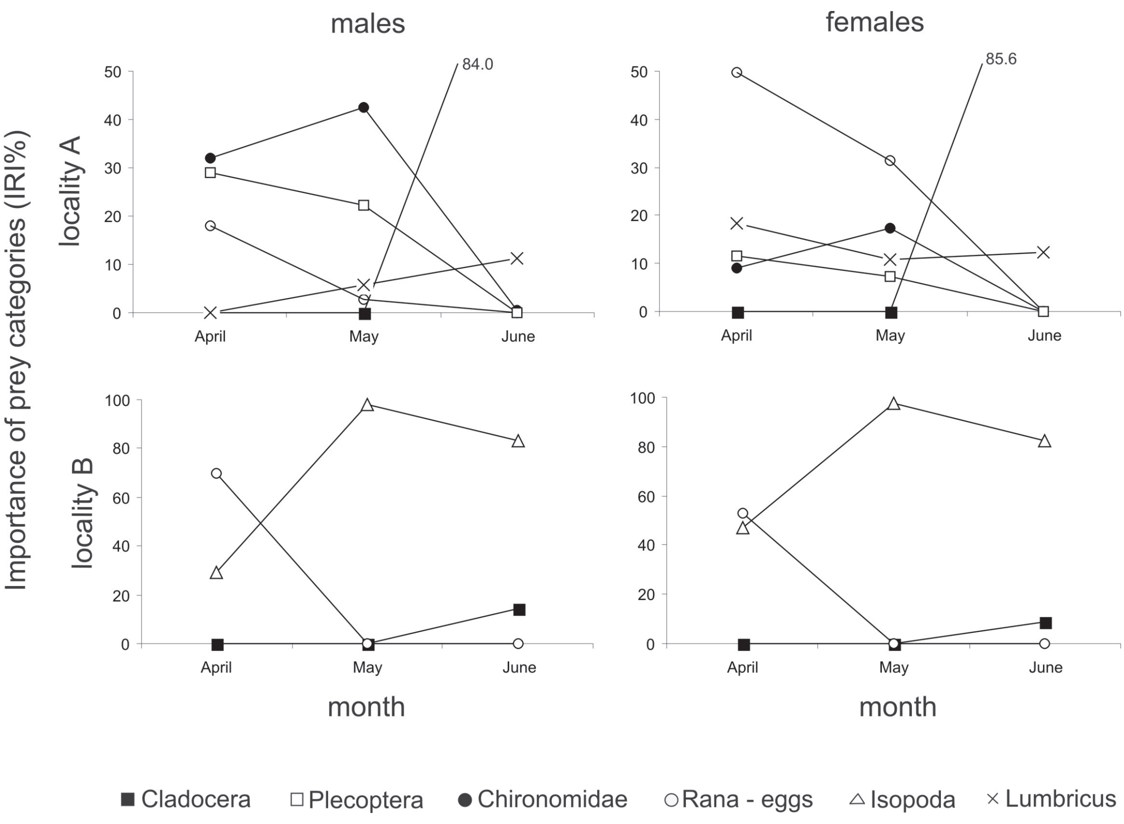

Fig. 1. – Changes in values of the index of relative importance (IRI%) of the main prey categories consumed by alpine newts.

TABLE 2

Differences in the number of consumed prey items between sexes of alpine newt from locality B (data from the months April, May, June pooled).

Prey	M mean (min.-max.)	F mean (min.-max.)	Z	P
Turbellaria	0.00	0.03 (0-1)	1.15	0.25
Oligochaeta	0.02 (0-1)	0.10 (0-1)	1.75	0.08
Gastropoda	0.00	0.00		
Bivalvia	0.00	0.00		
Arachnida	0.00	0.05 (0-2)	1.15	0.25
Cladocera	0.43 (0-4)	0.56 (0-7)	0.00	1.00
Cyclopoida	0.11 (0-2)	0.10 (0-1)	0.41	0.68
Isopoda	**1.11 (0-6)**	**1.85 (0-6)**	**2.66**	**0.01**
Ephemeroptera larvae	0.00	0.00		
Ephemeroptera pupae	0.02 (0-1)	0.00	-0.83	0.41
Plecoptera nymfa	0.02 (0-1)	0.00	-0.83	0.41
Megaloptera larvae	0.00	0.00		
Trichoptera	0.00	0.05 (0-2)	1.66	0.10
Lepidoptera	0.00	0.00		
Culicidae pupae	0.09 (0-1)	0.05 (0-1)	-0.45	0.65
Chironomidae larvae	0.07 (0-1)	0.03 (0-1)	-1.01	0.31
Chironomidae pupae	0.02 (0-1)	0.00	-0.83	0.41
Coleoptera	0.02 (0-1)	0.03 (0-1)	0.22	0.83
Rana eggs	0.46 (0-9)	0.23 (0-9)	-1.48	0.14

amphibian eggs (DENOËL & ANDREONE, 2003; DENOËL & DEMARS, 2008) are reported as the prey types most consumed. In the populations of the Czech Republic, important alpine newt prey include *Lumbricus*, Cladocera, Isopoda, *Rana* eggs and Ostracoda (RULÍK, 1993; KOPECKÝ et al., 2014).

The qualitative composition of prey items and their importance in the different sexes is constant within the species; in other words, males and females consume generally the same prey categories. Even *Lumbricus*, which was the largest prey type in our samples, was consumed by both sexes, regardless of the fact that females were about 15 % larger than the males at both localities (KOPECKÝ et al., 2012a).

Generally, female alpine newts consumed more prey items than males during the breeding period (KOPECKÝ et al., 2012a). This was especially obvious for prey categories with the highest importance at the particular localities – *Rana* eggs at locality A and Isopoda at locality B. Females also had a narrower niche breadth at both localities and during the whole aquatic season. Both these findings suggest that females are more sensitive to the trade-off between energy intake (from prey) and expenditure (spent while searching and hunting for prey). The metabolic cost of reproduction is probably higher for female alpine newts than for males, as was discovered in the case of ambystomatid salamaders (FINKLER & CULLUM , 2002).

Trophic niche separation can be achieved by selection of different microhabitats, food resource partitioning or temporal segregation (SCHOENER 1974). Temporal segregation was not found in the species studied (MARTIN et al., 1989), both sexes exhibited diel flexibility in hunting activities (JOLY & GIACOMA, 1992). Using different microhabitats is often described as a mechanism for the coexistence of different newt species of various sizes at the same locality (JOLY & GIACOMA, 1992; COVACIU-MARCOV

TABLE 3

Levins' indices of niche breadth of alpine newts from the localities studied (A, B).

		April	May	June
locality A	males	0.277	0.217	0.018
	females	0.261	0.183	0.005
locality B	males	0.079	0.071	0.124
	females	0.057	0.057	0.103

et al., 2010). On a subtle scale, intersexual resource partitioning is probably the key mechanism of niche separation (YAMAGUCHI et al., 2003). Intersexual resource partitioning is comparable with coexistence and feeding habits of paedomorphs and metamorphs of the same newt species, especially in shallow ponds (DENOËL & ANDREONE, 2003; VIGNOLI et al., 2007). The relatively high prey niche overlap of sexes (as observed in this study) is explained as the consequence of high abundance and high availability of potential prey in an environment that obscures the competition relationship (GRIFFITHS, 1987). Due to absence of data about prey availability at the both localities studied, it is also possible that the diversity of preyed taxa was not sufficient to reveal general difference in prey selectivity of the sexes.

At locality B the trophic niche overlap was stable and high over the three months, while at locality A it was generally lower, especially during April and May. The diversity of taxa consumable by newts was probably higher at locality A where newts caught prey from 18 taxa, while at locality B prey were consumed from only 14 taxa. There was a comparatively high prey niche overlap in June, especially at locality A where both sexes consumed Cladocera almost exclusively.

Newts are traditionally recognized as generalist predators (FASOLA & CANOVA, 1992). Generalists are characterized by having a niche breadth higher than 0.5 (MACARTHUR & LEVINS, 1967). At both localities studied, niche breadth during each of the months was lower than 0.5. As is evident from changes in prey importance, newts opportunistically shift among preyed taxon, that is, utilize the most accessible prey in a given period. For example, the abundance of immobile and biomass-dense *Rana* eggs, as one of main categories at both localities under study, decreased as the reproductive season advanced. Conversely, at the end of the newt's reproductive period, newts ate more but smaller prey items, which appeared abundantly in ponds – mainly small crustaceans (this study) or aphids that fell on the water surface, as was found in a population from the Italian Alps (VIGNOLI et al., 2007).

The occurrence of the prey taxa is connected with the specific and diverse microhabitats in ponds (water surface, water column, aquatic plants, muddy bottom, etc.) (DENOËL & JOLY, 2001), so newts must be able to switch between several hunting strategies and capture modes. Although there is no direct evidence from field studies, newts may search for food on land even during the breeding season, for example during movements between reproductive ponds (KOPECKÝ et al., 2010; KOPECKÝ et al., 2012b). Despite extensive morphological and physiological changes, newts can capture prey in the terrestrial environment due to behavioural plasticity, which compensates for the morphological constraints imposed by this water-terrestrial transition (HEISS et al., 2013). Hence niche breadth changes considerably during the breeding phase of newts.

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Mapping bird assemblages in a Mediterranean urban park: Evidence for a shift in dominance towards medium-large body sized species after 26 years

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ABSTRACT. We assessed the structure of the breeding bird assemblage in a Mediterranean urban park in 2012, and compared it with data gathered in the same area in 1986. Since 1986, Wryneck (*Jynx torquilla*) territories have disappeared from the study area, while breeding pairs of Green Woodpecker (*Picus viridis*) and the introduced Rose-ringed Parakeet (*Psittacula krameri*) have colonized the park. We observed a significant decrease in density of the Italian Sparrow (*Passer italiae*) and a significant increase in Starling (*Sturnus vulgaris*). At the assemblage level, overall bird densities decreased but total bird biomass increased due to the increase in density of (often cavity-nesting) medium to large body sized species (such as woodpeckers, Rose-ringed Parakeet and Starling). A presumed increase in mature tree availability and in predation by synanthropic species (e.g. crows) may explain the high density and biomass of primary and secondary cavity nesters characterized by medium-to-large body sizes. The decline of Sparrows and Wryneck may reflect the decreasing trend at the continental scale.

KEY WORDS: mapping method, long time span, *Jynx torquilla*, *Psittacula krameri*, *Sturnus vulgaris*, *Passer italiae*

INTRODUCTION

Urban parks have been considered important green areas that, embedded in anthropized landscapes, host specific bird assemblages (Beissinger & Osborne, 1982; Fernández-Juricic & Jokimäki, 2001; Kelcey & Rheinwald, 2005; Chace & Walsh, 2006; Clergeau et al., 2006; Ortega-Álvarez & MacGregor-Fors, 2009; Ramalho & Hobbs, 2012). In this sense, several studies have evidenced their peculiar composition and structure (for central and northern Europe: De Graaf et al., 1991; Jokimäki & Suhonen, 1993; for Mediterranean: Fernández-Juricic, 2000; Sorace & Gustin, 2008; Fraissinet & Fulgione, 2008; see also for review: McKinney, 2002; Clergeau et al., 2006). In particular, in the last decades several generalist synanthropic species, both native and non-native, have increased in density in urban areas, changing the total richness and inducing evident turnovers in species assemblages (Sorace & Gustin, 2008). Among them, species such as parakeets, starlings and crows may be considered as urban exploiter species, while many specialized species have shown a progressive decline (Palomino & Carrascal, 2006). Although these changes are documented in literature (Kelcey & Rheinwald, 2005; McCaffrey & Mannan, 2012), empirical research comparing bird assemblages over large time spans is scarce, at least for Mediterranean urban parks.

In 1986, breeding birds were censused in a large urban park of central Italy (Rome) using a mapping method (Battisti, 1986). After 26 years (2012) we carried out a comparable study in the same site, using the same protocol by the same researcher (CB). This comparison allowed us to detect changes in density at species level, and in structure at assemblage level over this long time span (1986-2012). Data were analyzed using a bi-variate metric of diversity (Abundance/Biomass curves) based on a comparison of cumulative species abundance and biomasses, used to detect structural changes at community level (Warwick & Clarke, 1994; Magurran, 2004).

MATERIAL AND METHODS

The area studied was inside the Villa Doria Pamphili (Rome, central Italy), a large historical urban park (120 hectares, 50 m a.s.l.; Site of Conservation Interest 'Habitat' Directive 92/43/CEE; Code IT6003052; 41°53' N, 12°27' E). Inside the park, we identified a study area (17.96 ha wide) characterized by native (*Quercus ilex*, *Q. pubescens*, *Q. petrae* and, secondarily, *Ulmus campetris, Laurus nobilis, Cercis siliquastrum*) and ornamental trees (*Pinus pinea, Cedrus libanotica*, *Aesculus hippocastanum, Robinia pseudoacacia, Cupressus* sp., *Ailanthus altissima*) surrounded by mowed grasslands (mainly Graminaeae, Malvaceae, Compositae; Celesti-Grapow, 1995).

Breeding bird assemblages were monitored by means of a mapping census method (Bibby et al., 2000; Sutherland 2006). During the 2012 breeding seasons (March-June), a number of periodic visits (n = 12) were carried out with a sampling effort (25 hours of field sampling) comparable to the research effort carried out in 1986 (12 visits; 25 hours). During each visit, the observer collected data following a non-linear transect (about 2,500 m-long; speed: 1.5 km/hour) in the early morning (07.00 -10.00 a.m.) covering all the study area. Contacts (i.e. records of each individual breeding bird) were noted on a local map (scale 1:2,000, Technical Regional Map; Regione Lazio, 1990). The primary and secondary tree vegetation was the same as in 1986, and no evident changes in their relative abundance had occurred when compared to 2012. No form of wood coppicing or clearing had been carried out in the period between the two surveys (Celesti Grapow, 1995; Carbone & Frassineti, 2001; Ricotta *et al.,* 2001).

Species-specific maps were created and species-specific territories were obtained following the clustering procedure described in Bibby *et al.* (2000). We considered a "territory" as a range area where a territorial species pair was considered to breed (Bibby *et al.*, 2000). Due to the limited vocalizations or territoriality of some species (e.g. sparrows *Passer* sp., Hooded Crow *Corvus cornix*, Rose-ringed Parakeet *Psittacula krameri*) an estimated value of density of such species was drawn from the counting of the individuals and checking for their nests. Species with crepuscular or nocturnal activity (e.g. Strigiformes and Caprimulgiformes) and individuals flying very high (> 25 m) were not considered. For nomenclature we refer to Italian Sparrow *Passer italiae* since Hermansen *et al.* (2011) established definitively that this species is a stabilized hybrid.

We analysed data at assemblage and species levels for both time periods. We refer to the term "assemblage" to indicate a set of taxonomically related species that co-occur at a given time and spatial scale in a site (Verner, 1984; Fauth et al., 1996). The following parameters were calculated: (i) species richness, as the number of species occurring in the study area for the overall assemblage (STot); (ii) total number of breeding pairs (Ntot) and breeding pair density (D), this last expressed as number of territories (i.e. breeding pairs)/10 ha and calculated for each species and all species (DTot); (iii) relative frequency for each species both in abundance (f_A as the ratio: D/DTot; species with $f_A > 0.05$ were considered dominant species; Turček, 1956) and biomass (f_B as the ratio: species-specific body weight/total biomass); (iv) Shannon diversity index (H'; Shannon & Weaver, 1963, as $H = -\Sigma f_A \ln f_A$); (v) total biomass (TB; both at species and assemblage level), corresponding to the sum of body weight of all censused individuals, expressed in g). To calculate the biomass values, mean body mass values were obtained from CRAMP & SIMMONS (1977, 1980, 1983), CRAMP (1988) and CRAMP & PERRINS (1993). For each species in the assemblage, we additionally obtained their cumulative frequency for abundance and for biomass. We then ranked the cumulative frequencies from the most to the least important along the x-axis in a Cartesian space in order to obtain two curves, for cumulative abundance and for biomass (ABC curves; WARWICK, 1986; MAGURRAN, 2004). In particular, species abundance curves indicate the relative distribution of the spatial niche of the species (using abundance as a proxy), while biomass curves indicate the relative distribution of the energy flow in the assemblage, according to the trophic resources used by species (MAGURRAN, 2004). The ABC approach has been applied in several animal assemblages (PENCZAK & KRUK, 1999; MAGURRAN & PHILLIP, 2001; PRETE et al., 2012), but rarely in birds (e.g., BENASSI et al., 2009).

The comparison between relative frequencies in abundance of each species in paired years (1986 *vs* 2012; only for species with at least a census of > 5 total pairs) was tested using χ^2 test. We performed the Kolmogorov-Smirnov test (two-tailed) to compare the diversity indices and the frequency distribution between curves (1986 *vs* 2012). We used statistical package Primer version 4.02i for Windows and SPSS 13.0 for Windows (SPSS Inc. 2003). Significance levels were set at $p < 0.05$. We checked for data reliability (controlling for standardization, replication, data-independence) following BATTISTI *et al.* (2014).

RESULTS

In the 2012 breeding season, we sampled 20 breeding species (compared to 23 in 1986; Table 1). Comparing data between years, in 2012 we no longer observed Wryneck *Jynx torquilla,* which had been present in 1986, and we recorded pairs of Green Woodpecker *Picus viridis* and Rose-ringed Parakeet for the first time in 2012. We observed a significant decrease in frequency of abundance (P<0.05) for Italian Sparrow and Wryneck and an increase for Starling *Sturnus vulgaris* (P<0.05) and Rose-ringed Parakeet (P<0.01; χ^2 test; Table 1). At assemblage level, we also observed contrasting trends among parameters: from 1986 to 2012, the total density decreased and the total biomass increased (Table 1). In 2012, 36.8% of total biomass was related to only two cavity nester species (Starling and Rose-ringed Parakeet). Cavity nesters markedly increased their total frequency in biomass (1986: 0.353; 2012: 0.549).

Diversity index H' showed a weak, not significant change between 1986 and 2012 (H' = 2.73 *vs* H' = 2.69; Z = 0.384, P = 0.998; Kolmogorov-Smirnov test). The ABC curves show that (i) biomass curves are higher when compared to abundance curves in both years; (ii) curves for 2012 (abundance and biomass) cumulate early when compared to curves for 1986. Differences between biomass cumulative curves are significant (Z = 1.991, P = 0.003; Kolmogorov-Smirnov test; Fig. 1).

DISCUSSION

Our data show that over a 26-year period, quantitative changes in breeding bird assemblages occurred in our study area, but these differences were mainly driven by the population trends of a limited set of species. Some species, occurring in 1986, showed a significant decline in their frequency (such as sparrows) or were not recorded at all in 2012 (Wryneck). Other species newly appeared (Rose-ringed Parakeet, Green Woodpecker) or significantly increased both in density and in their frequency (Starling). Rose-ringed Parakeet, absent in 1986 but dominant in 2012, was introduced into European urban areas in the 1980s (CZAIJKA *et al.*, 2011) and shows a recent and strong expansion in Italy (MORI *et al.*, 2013). This parakeet is considered a new invasive alien species occurring in urban European ecosystems where it can compete

TABLE 1

Breeding bird species of the Villa Doria Pamphili urban park (Rome, central Italy) for 1986 (data from Battisti 1986) and 2012 (original data). C = cavity nester species. Ntot = total number of breeding pairs, D = species density (pairs/10 ha), f_A = relative frequency in abundance (in bold, the dominant species: fi>0.05), f_B = relative frequency in biomass; TB = total biomass (in g). Values of χ^2 (comparison between frequency in abundance) were reported only for species with at least >5 total pairs censused; * = P<0.05; ** = P<0.01).

	1986					2012					
Species	Ntot	D	f_A	TB	f_B	Ntot	D	f_A	TB	f_B	χ^2 test
Sylvia atricapilla	38.5	21.4	**0.177**	643.2	0.087	28	15.6	**0.166**	468.6	0.057	0.125
Troglodytes troglodytes	30.5	17	**0.14**	271.7	0.037	19.5	10.9	**0.116**	173.5	0.021	0.297
Passer italiae (C)	15.5	11.1	**0.092**	668.4	0.091	3	1.7	0.018	100.6	0.012	4.917*
Serinus serinus	15.5	8.6	**0.071**	155.3	0.021	4	2.2	0.023	40.2	0.005	3.657
Turdus merula	15	8.3	**0.069**	1219.1	0.166	10	5.6	**0.06**	817.8	0.099	0.047
Parus major (C)	14	7.8	**0.064**	280.4	0.038	13	7.2	**0.077**	260.2	0.032	0.047
Fringilla coelebs	12.5	7	**0.057**	292.3	0.04	7.5	4.2	0.045	174.4	0.021	0.114
Sturnus vulgaris (C)	10	5.6	0.046	946.9	0.129	20	11.1	**0.118**	1883	0.228	4.711*
Cyanistes caeruleus (C)	9.5	5.3	0.044	105.8	0.014	15	8.4	**0.089**	166.7	0.02	1.728
Jynx torquilla (C)	8.5	4.7	0.039	312.2	0.042						5.354*
Erithacus rubecula	6.5	3.6	0.03	115.8	0.016	8	4.5	0.048	143.3	0.017	0.177
Certhia brachydactyla (C)	5	2.8	0.023	44.5	0.006	5.5	3.1	0.033	48.7	0.006	0.131
Regulus ignicapilla	5	2.8	0.023	27.8	0.004	7	3.9	0.041	38.7	0.005	0.441
Passer montanus (C)	5	2.8	0.023	127.9	0.017						2.349
Carduelis chloris	4.5	2.5	0.021	120.5	0.016	3.5	1.9	0.02	93.9	0.011	
Luscinia megarhynchos	2	1.7	0.014	66.8	0.009	1	0.6	0.007	21.9	0.003	
Cettia cetti	3	1.7	0.014	46.8	0.006						
Corvus cornix	3	1.7	0.014	1686.7	0.229	3	1.7	0.018	1657.3	0.201	
Carduelis carduelis	2	1.1	0.009	35.5	0.005	4	2.2	0.023	71.9	0.009	
Sylvia melanocephala	2	1.1	0.009	26.6	0.004						
Muscicapa striata	2	1.1	0.009	32.2	0.004						
Dendrocopos major (C)	1.5	0.8	0.007	117.6	0.016	3	1.7	0.018	245.5	0.03	
Aegithalos caudatus	1	0.6	0.005	7.8	0.001	2	1.1	0.012	14.5	0.002	
Picus viridis (C)						3	1.7	0.019	668.2	0.081	
Psittacula krameri (C)						9	5	**0.053**	1152.6	0.14	8.844**
Total	212	121.1	1	7351.8		169	94.1	1	8241.4		

with many primary and secondary hole-nesting species (DODARO & BATTISTI, 2014). The Green Woodpecker has shown a moderate increase at the continental scale in recent years (GREGORY *et al.*, 2007), and the appearance of the species in our study area corroborates such a continent-wide increase. Starlings are among the most common secondary cavity-nesters in Europe (FEARE, 1984; KOENIG, 2003), breeding in central Italy from the 1970s and nowadays occurring almost everywhere as a breeder (CECERE *et al.*, 2005). Interestingly, while starlings are known to compete for nesting cavities with Rose-ringed Parakeets (DODARO & BATTISTI, 2014, see also STRUBBE & MATTHYSEN, 2007; 2009a; 2009b; CZAIJKA *et al.*, 2011; NEWSON *et al.*, 2013), both species have become more common in our study area, probably because tree maturation may have increased the availability of suitable nesting cavities. Similarly to the European Sparrow, the

Italian Sparrow significantly declined from 1986 to 2012, supporting the evidence of its general decline in the last decade (SUMMER-SMITH, 2003; BRICHETTI *et al.*, 2008; CAMPEDELLI *et al.*, 2012).

Different factors and processes at different scales may act to determine the observed patterns, as stated for urban parks in non-Mediterranean contexts (e.g. JOKIMÄKI, 1999). At the local scale, the availability of large native and ornamental trees (and their maturation over the last 26 years), combined with the ability of Rose-ringed Parakeets to enlarge cavities for nesting (ORCHAN *et al.,* 2013), may explain the occurrence and high density of several medium-large cavity nesters (ANGELSTAM & MIKUSIŃKI, 1994; MIKUSIŇSKI *et al.*, 2001; PASINELLI, 2007; STRUBBE & MATTHYSEN, 2007; ZANGARI *et al.*, 2013), while the decline of sparrows and Wryneck follows a larger scale (continental) process (GREGORY *et al.*, 2007; REIF, 2013).

Although the number of species slightly decreased from 1986 to 2012, the biomass at assemblage level increased because of an increase in the density of medium-large bodied species (such as woodpeckers, Rose-ringed parakeet*,* Starling). The ABC curves (Fig. 1) emphasize the different ecological roles that the biomass and abundance parameters have at community level. When compared to 1986, assemblage energy flow has been progressively controlled by a set of medium-large bodied species. Interestingly, the two more common medium-large species (Rose-ringed Parakeet and Starling) are also cavity nesters, and total frequency in biomass of cavity nesters has markedly increased from 1986 to 2010. Presumably, in urban habitats, where egg and juvenile predation by crows and other synanthropic predators (rats, feral cats) can be very high, this ecological trait could be selectively favored (CROCI *et al.,* 2008; JOKIMÄKI & HUHTA, 2000; JOKIMÄKI *et al.,* 2005; SERESS & LIKER, 2015).

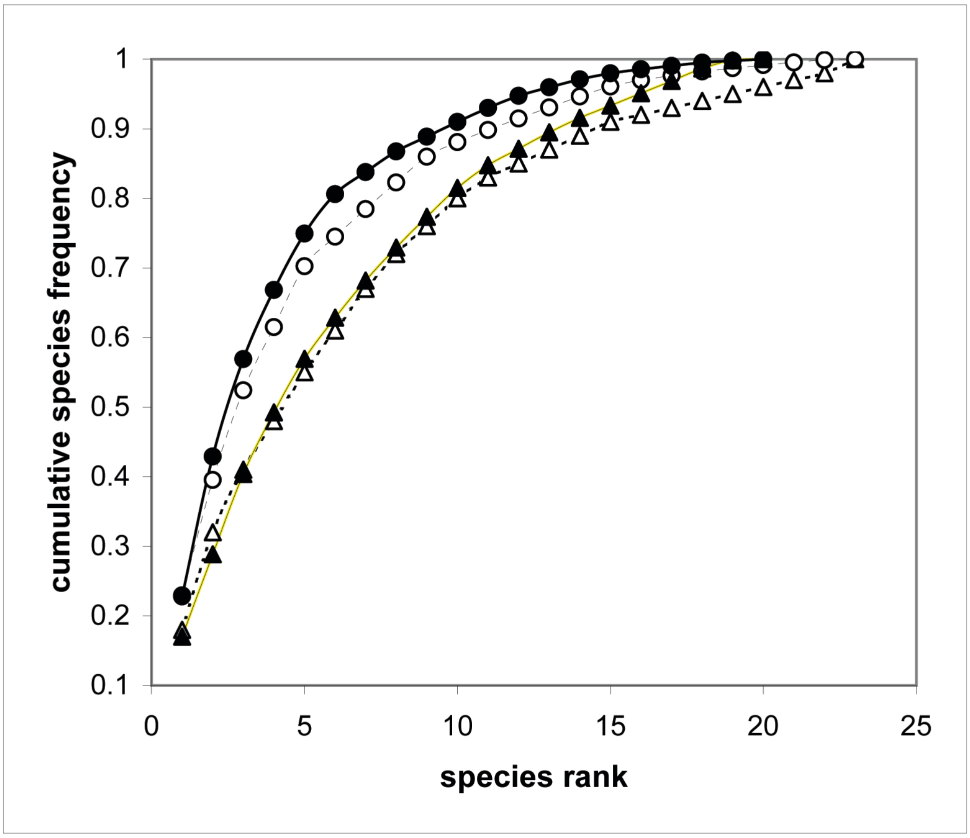

Fig. 1. – Abundance-Biomass Comparisons (ABC curves) for the breeding bird assemblages in Villa Pamphili urban park (central Italy). Circles: biomass cumulative frequency; triangles: abundance cumulative frequency (white and dashed lines: 1986; black and continuous lines: 2012).

When abundance and biomass curves are compared, we then may obtain information on the structure of assemblages, i.e. if dominated by small bodied and highly abundant species or by large bodied and less abundant species. When biomass curves cumulate before the abundance curves, it is an indication that a higher number of relatively large bodied species occur in a more mature assemblage (MAGURRAN, 2004). In our case, in both years we observed biomass curves cumulating before the abundance curves. Nevertheless, this trend appears to be more prominent in 2012 when the biomass curve cumulated significantly earlier when compared to data sampled 26 years earlier. Early-cumulating biomass curves may indicate that more individuals with larger body size (and dominant in biomass) occur in the assemblage. Following this model, we observed that bird assemblages progressively changed toward species with larger body size and lesser abundance.

It should however be noted that our data may be affected by some weaknesses: i) it has been evidenced that the sampling performance of the same observers changes during large time spans (observer effect; see MAGURRAN *et al.*, 2010); ii) our data belong to a single urban park and, therefore, may be affected by local environmental constraints and casual factors; iii) within our time span, we carried out the study in two years only. Nevertheless, our data have some points of strength: i) this is the first study carried out over a large time span in a Mediterranean urban park that highlights changes in assemblage structure with a shift toward medium-large bodied species; ii) we confirmed, at a local level, changes in density matching analogous trends at a larger scale: i.e. an increase for Rose-ringed Parakeet (see MORI et al., 2013; PYŠEK & HULME, 2011) and a decrease for Italian Sparrow and Wryneck (see GREGORY et al., 2007); iii) for Starling, a species stable or moderately declining at continental scale (GREGORY et al., 2007; FREEMAN et al., 2007), we observed an increase in density matching the national trend (CAMPEDELLI et al., 2012); iv) finally, at assemblage level, we confirmed the general pattern described from REIF (2013) at continental scale, i.e. smaller body sized species are declining while species with larger body sizes have increased or at least shown a less negative trend.

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Movements and habitat use of the invasive species *Lithobates catesbeianus* in the valley of the Grote Nete (Belgium)

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ABSTRACT. Nine adult American bullfrogs (*Lithobates catesbeianus*) were tagged with an internal radio transmitter and tracked during one year in the valley of the Grote Nete (Belgium). The mean ± SD core range area (KDE50) was 15.00 ± 22.41m^2. The home range area (KDE95) had a mean ± SD of 429.78 ± 510.97m^2. Shores of larger eutrophic ponds and small temporary pools in alluvial forest were chosen as habitat. The total area used (MCP95) had a mean of 11,086.73 ± 12,239.00m^2. The study revealed a mean action radius of 270.78 ± 199.17m and individuals moved up to 742m in a single displacement. These results show that the dispersion of the American bullfrog in a valley system such as the Grote Nete can proceed very rapidly. A positive correlation between weight and distance covered within one movement was found, which could suggest that dominant individuals are capable of covering greater distances in search of optimal habitat for reproduction, foraging or hibernation.

KEY WORDS: invasive species, SAC, radio telemetry, American bullfrog, *Rana catesbeiana*

INTRODUCTION

Invasive species are a worldwide threat for native biodiversity and are a major cause of the extinction of species (Clavero & Garcia-Berthou, 2005). The American bullfrog (*Lithobates catesbeianus* Shaw, 1802) is listed on the IUCN's list of the world's hundred worst invasive alien species because of its invasive character and its ecological impact (Lowe et al., 2000).

The natural range of the American bullfrog spans a wide latitude, extending north to Canada (Nova Scotia, New Brunswick, southern Quebec and southern Ontario) and south to central Florida and north-eastern Mexico. This vast natural range illustrates the species' flexible life history and broad climatic and ecological amplitude, which contributes to its success as an invasive alien species (D'Amore, 2012). In the 20th century this species was introduced in aquaculture as a biological control agent or for ornamental purposes (Jennings & Hayes, 1985). It has since then been partly responsible for the decline of populations of native species (Adams & Pearl, 2007).

In Belgium the invasive exotic American bullfrog is widely spread in the valley of the Grote Nete, where the population inhabits over 400 ponds in an area of 100km^2. The valley of the Grote Nete is assigned as a Special Area of Conservation (SAC) for the Habitats Directive and consists of 4,280 hectares of alluvial forests, eutrophic ponds, marshes and mesotrophic meadows (Agentschap voor Natuur en Bos, 2012). Within this system the invasive American bullfrog has dispersed from its initial point of invasion (Zammelsbroek) upstream and downstream along the river Grote Nete.

The American bullfrog is an opportunistic feeder and feeds on larger invertebrates, fishes, indigenous amphibians, young reptiles and their own larvae and sub-adults (Leivas et al., 2012). This species is also a vector of the chytrid fungus *Batrachochytrium dendrobatidis* (Longcore, PESSIER &. NICHOLS, 1999). A recent study demonstrated that 63.4% of the adult bullfrogs and 20.5% of the larvae in Belgium are infected (PASMANS & MARTEL, 2012). This chytrid fungus contributed to the total extinction of a population of amphibia in Brazil (SCHLOEGEL et al., 2010). The presence and abundance of this invasive species in the valley of the Grote Nete forms, consequently, a potential threat to local biodiversity in this European SAC.

The American bullfrog generally prefers still, deep water habitats with rooted floating vegetation and open shoreline vegetation (FULLER et al., 2010). Permanent wetlands form a possible indicator of both bullfrog occupancy and the presence of a reproducing population. Moreover, the distance of a wetland to the nearest lake or pond, as well as the amount of wetland area within a 1km-buffer, is positively associated with bullfrog presence. The occurrence of waterway corridors, whether or not human made, also favours the dispersion of this species across the landscape (PETERSON et al., 2013).

Local field data on the invasive bullfrog are fragmented but are important to evaluate the impact of this biological invasion. Knowledge of their local behaviour, dispersion rate and movements are essential to optimise control methods and actions. To gain a better insight into the activity, home range and dispersion of this species, a telemetric study in the upstream part of the valley was set up.

MATERIAL AND METHODS

Transmitter

Due to the difficulty in obtaining wild caught American bullfrogs, only nine adult bullfrogs were tagged with an internal transmitter and tracked during one year.

Only sexually mature frogs with a length of more than 11cm snout to stout (BRUNEAU & MAGNIN, 1980) and a weight over 180 gram were selected (Table 1) to gain insight into the dispersion and reproductive behaviour of adult individuals.

Implantable transmitters are most suitable because they interfere less than external transmitters with the long-term behaviour and lifespan of the animal (MIAUD et al., 2000). Radio-transmitters should not exceed more than 10% of the body mass of the animal, but many authors suggest an even more conservative limit of 5% (RICHARDS et al., 1994). Therefore, the transmitter R1170 (ATS Inc., Isanti, MN, USA) was used. It has a weight of 4 grams and a lifespan of the battery of approximately 440 days. Pulse speed and -length was at 30ppm and 15ms respectively. To localize the animals a 3-element Yagi antenna (Bluesky Telemetry, Perthshire, UK) and a R410 receiver (ATS Inc., Isanti, MN, USA) were used.

Study area and implanting procedure

Five male and four female adult American bullfrogs were caught in May 2011 with fykes (0.8m diameter, 1cm mesh) in the same pond in the valley of the Grote Nete (51°8' N, 05°8' E) (Fig. 1). The eutrophic pond has a surface of 4,280m^2 where the shores are dominated by *Phragmites australis*, *Typha latifolia*, often with overhanging shoreline vegetation such as *Rubus* spec. and *Carex* spec. The surrounding habitat consists of alluvial forest with small puddles and larger ponds, crossed by the river Grote Nete.

The frogs were weighed and measured from snout to stout (Table 1) and anesthetised in tricaine methanesulfonate (MS-222, 2g/l). A ventral cut of 1 cm in the abdominal left side was made in the skin and abdominal muscles. The transmitter was placed in the intraperitoneal cavity; muscle layer and skin were each closed with four sutures of absorbable Monocryl-plus (Ethicon, Somerville, NJ, USA). Finally the wound, with sutures, was covered with Vetbond skin glue (3M, St.Paul, MN, USA). After surgery, the animals recovered for 30 minutes in fresh pond water and were released at the site of capture.

Before the start of this experiment, a dummy transmitter was implanted in adult bullfrogs under laboratory conditions to evaluate the impact of the procedure. The animals recovered well and showed no visible disadvantage from the implant. Internal examination of the animals one month post implantation showed that the dummy transmitter was encapsulated by connective tissue around the intestines of the animal and did not impede the functioning of any of the organs.

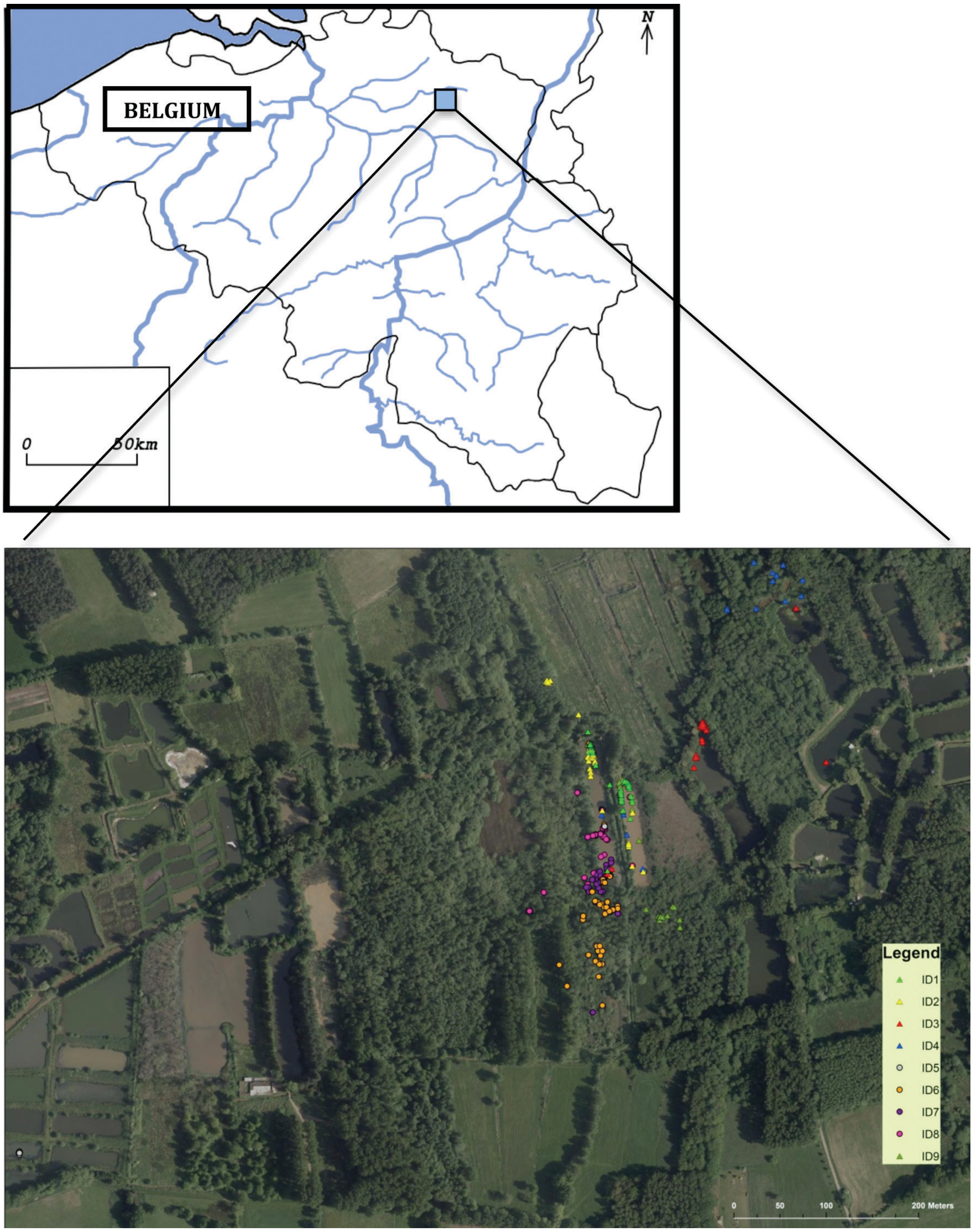

Fig. 1. – Localisation of the research area and the fixes of the tracked bullfrogs within the valley of the Grote Nete (ArcGIS 10).

TABLE 1

Characteristics of the radio-tagged bullfrogs, start and end of tracking period and number of fixes per individual.

Individual	Gender	Weight (g)	Length (cm)	Start date	End date	# Fixes
1	M	196	12.9	12/05/11	28/08/12	136
2	M	180	11.2	16/05/11	04/07/12	131
3	M	492	16.5	17/05/11	28/08/12	139
4	M	252	14	31/05/11	28/08/12	87
5	F	348	13.2	12/05/11	28/08/12	10
6	F	312	14.8	12/05/11	28/08/12	137
7	F	202	11.7	12/05/11	28/08/12	137
8	F	268	14	16/05/11	25/07/12	118
9	M	298	14.7	31/05/11	28/08/12	134

Radio tracking

During the months of May till September 2012 the position of each frog was determined by triangulation twice a week, alternately in the morning and the evening. This interval allowed an accurate estimation of home range in the fish *Barbus barbus* (Linnaeus, 1758), which is a far more mobile species than the American bullfrog (Baras, 1998). Every month a 24h-cycle was executed and frogs were tracked every hour, to get a better insight into total movements during day and night. From October till March, the period of winter torpor, the animals were localized once a month.

At the end of the tracking period, which lasted 16 months, only two animals could not be localized due to the life-end of the batteries (Table 1).

Data analysis

Data analysis was performed using ArcGIS Spatial Analyst 10 (Esri, Redlands, CA, USA) and HRT-tools for ArcGIS (Rodgers et al., 2007). To gain insight into the total area used by an animal the Minimal Convex Polygon 95% (MCP95) was calculated. Moreover, Kernel Density Estimates 50% (KDE50) and 95% (KDE95) were performed on the tracking results to define respectively the core range and home range of each individual bullfrog. For statistical analysis SPSS statistics 22 (IBM, Armonk, NY, USA) was used.

RESULTS

During the tracking period two individuals (nr. 4 and 5) were temporarily unable to be located, partly due to the inaccessibility of the habitat so fewer fixes were available (Table 1).

At the start there was no statistically significant difference in length and weight of the two sexes (Mann-Whitney U, p = 0.905 and p = 0.556 respectively).

Most individuals remained in the area where they were caught, but some dispersed further into the surrounding landscape using permanent ponds and marshes in alluvial forest or the Grote Nete as a guide line (Fig. 1). Mean convex polygons (MCP95) were calculated for the different individuals, and revealed a mean ± SD of 11,086.73 ± 12,239.00m^2 of total area used by adult American bullfrogs. To have a better idea of the more exact home range of these individuals, KDE95 was calculated (Table 2). The mean home range was 429.78 ± 510.97m^2,

TABLE 2

Results of the Kernel density analysis with habitat type for KDE50 and MCP 95% per individual. (A = permanent pond, B = swamp in alluvial forest).

	Fixed Kernel				MCP 95%
ID	**Core range (m²) KDE50**	**KDE50 Habitat**	**Home range (m²) KDE95**	**# of locations KDE95**	**Total range (m²)**
1	5.09	A	65.86	17	1286.91
2	11.06 - 15.24	A	365.21	9	3772.05
3	62.43	A	412.26	3	3656.12
4	4.8 - 61.63	B - A	1723.94	16	38413.84
5	68.66	A	148.72	1	23937.73
6	1.23 - 1.37 - 1.39 - 3.08 - 5.61	B	282.9	27	10455.06
7	0.47 - 1.35	B - A	59.67	25	5030.27
8	10.37 - 13.11	B -A	542.79	12	8128.63
9	0.81 - 5.10 - 12.25	A - A - B	266.66	11	5099.93
Mean ± SD	**22.64 ± 26.18**		**429.78 ± 510.97**		**11086.73 ± 12239.00**

with a mean of 13 ± 9 different locations used. To define the core ranges of the American bullfrogs KDE50 was determined (Table 2). Sixty seven per cent of the individuals showed more than one Kernel 50% position and the mean area was 15.0 ± 22.41m². The KDE50 habitats were examined and are either permanent ponds (habitat A) or swampy puddles in alluvial forest (habitat B) (Table 2).

Analysis of the MCP95, KDE50 and KDE95 showed no statistically significant differences in area occupied by male or female American bullfrogs (Mann-Whitney U, $p = 0.221$, $p = 0.462$ and $p = 0.462$ respectively). No statistically significant correlation was found between length, weight or sex of the individuals and their major choice of habitat, core and home range size.

To gain insight in the dispersion abilities of individual adult bullfrogs the total distance, maximal distance from point of release and maximal distance in a single movement were calculated (Table 3). The total distance travelled during the tracking period varied greatly between individuals, with a mean ± SD of 1,152.23 ± 348.56m. The maximal distance travelled from the release point had a mean ± SD of 270.78 ± 199.17m. Also the maximal distance covered in a single movement varied greatly between individuals and showed a mean ± SD of 248.70 ± 202.34m (Table 3). All the variables analysed in Table 3 varied greatly between individuals.

There was no statistically significant difference between sexes in the maximal distance from the point of release and in a single movement (Mann-Whitney U, $p = 0.806$ and $p = 0.624$ respectively). No statistically significant correlation was found between length of the animal and maximal distance from point of release and distance travelled in a single movement (Spearman, r = 0.433 and $r = 0.360$). Weight of the bullfrogs was not correlated with maximal distance from point of release (Spearman, $r = 0.142$), but a significant correlation was found with the maximal distance covered in one single movement (Spearman, r = 0.683).

TABLE 3

Overview distances per individual.

Individual	Total distance (m)	Max. distance from release point (m)	Max. distance in a single movement (m)
1	878.00	152.06	130.48
2	1177.26	140.13	118.91
3	1125.52	345.90	353.16
4	1089.48	414.51	295.54
5	814.08	726.54	742.21
6	1818.31	145.59	135.61
7	1621.98	233.61	167.71
8	861.57	122.77	138.94
9	983.85	155.93	155.71
Mean ± SD	**1152.23 ± 348.56**	**270.78 ± 199.17**	**248.70 ± 202.34**

DISCUSSION

The KDE50 analysis shows that adult American bullfrogs in the valley of the Grote Nete had a core range (KDE50) with an average size of 15.00 ± 22.41 m^2. Habitats were located in the littoral zone of ponds or under bushes at the edge of pools or puddles in the alluvial forest. Some of the frogs temporarily changed location, which resulted in more than one KDE50 area for the specimen (Table 2, Fig. 2). A possible explanation is that individuals who had their core range within the alluvial forest, went to larger ponds during the reproductive season and returned to their initial spot later. This behaviour has also been observed in Southwest France where the bullfrogs reached the reproductive pond in June after spending two months in a flooded area (BERRONEAU et al., 2007). Moreover, within the summer feeding habitat the frogs may change position in search for food and shelter. The home ranges (KDE95) of the frogs in this study, composed of a number of distinct spots, suggest that they used different suboptimal habitats for shelter and foraging. During the 12 and 24 hours tracking sessions the individuals hardly moved, which can be explained by assuming that they found their food and shelter within their KDE95 spots and did not actively search for prey, especially in the ponds where bullfrog larvae and topmouth gudgeon (*Pseudorasbora parva* TEMMINCK & SCHLEGEL, 1846) prey were very abundant near the shelters.

The movements of the nine individual frogs revealed that only two types of habitats (Table 2) were used, one is the shoreline of the ponds dominated with *Phragmites australis*, *Typha latifolia*, with overhanging shoreline vegetation such as *Rubus spec.* and *Carex spec.* (habitat A). The other habitat consists of brooks with shallow pools or puddles within alluvial forest (habitat B). In a Canadian study, the mean activity radius, used as an index of home range size (in Ontario, Canada), was 21.40m^2 (CURRIE & BELLIS, 1969). The authors stated that the home range size may be reduced at high densities. In this Canadian study the bullfrog spots were almost all located in water in spite of occasional visits to land. These findings differ from our results of the adult bullfrogs in the valley of the Grote Nete, which have shown a home range (KDE95) of 429.78 ± 510.97m^2. We should keep in mind that our study was carried out over a longer period than the one-month one in Ontario (Canada). Moreover, the larger home range in our study also indicates a lower density in the examined area as documented by (CURRIE & BELLIS, 1969) and/or a difference in behaviour between the frogs in Canada and Belgium. The density of American bullfrogs in adjacent ponds in the valley of the Grote Nete has been examined, and showed an estimate of 46 individuals/ha water surface (LOUETTE et al., 2013) while the bullfrogs in Ontario (Canada) had a density of 272-420 individuals/ha (CURRIE & BELLIS, 1969). It is also possible that bullfrogs of an invasive population demonstrate increased dispersive behaviour.

Seasonal pools are a part of the bullfrog habitat complex, providing the population with food, refugium and stepping stones (GAHL et al., 2009). This author stated that the use of these pools varied for sex and age category, and that males were often found in seasonal pools before the reproduction season. This could not be confirmed from our study in the valley of the Grote Nete, where 60% of the males had their home range (KDE50) exclusively in permanent ponds and 40% in seasonal puddles and ponds. Additionally no significant correlation was noticed between the sex and the amount of KDE50 in the shorelines of permanent ponds. However, males examined in our preliminary study tended to prefer the shore of permanent ponds while females were equally divided between both habitats. Likewise, no statistical correlation was found between the area of a KDE50 spot and the amount of KDE50 spots in alluvial forest. However, a trend was notable, suggesting that smaller but more spots are occupied when the KDE50 habitat is located in alluvial forest. Possibly this is due to a lower abundance of food or quality of shelters in this alluvial forest compared to the shores of large permanent ponds. Further research on the movements of a higher number of adult bullfrogs with telemetry could give better insight in the habitat use of both sexes.

As for hibernation, a study in Summit County USA showed that the bullfrogs favour relatively shallow (<1m) sites with algae and cattails, fed by small streams (STINNER et al., 1994). A habitat model made for the American bullfrog showed that the suitability of a wetland as winter cover can be expressed as a combination of the winter water depth and the relative amount of silt in the bottom substrates (GRAVES & ANDERSON, 1987). Another telemetric study in France revealed that 80% of the individuals of the American bullfrog hibernated under mulch in wooded area (BERRONEAU et al., 2007). The habitat choice for hibernation in this study was equally divided among the individuals. Fifty per cent of the investigated bullfrogs favoured the littoral zone of large permanent ponds, while the others preferred the wet soil in the alluvial forest. During the winter period only one individual showed some smaller movements, but in general

Fig. 2. – KDE50 and KDE95 locations (red and green, respectively) for the individual nr.1, 6 and 9 [see the online version for the colour figure].

the hibernation positions were maintained during this season. These winter localities correspond with the KDE50 positions, which suggests that the core range habitat is suited for both summer foraging and shelter as well as for winter hibernation. The fact that individuals showed movements during the cold season makes it clear that they are not fully torpid during the whole winter season, but that they probably have torpid periods alternated with short active moments so they can avoid unfavourable conditions or forage. In Southwest France several individuals were reported active during winter and they carried out important movements (BERRONEAU et al., 2007).

Analysis of the movements made by the tracked adult bullfrogs in our study show that long distances can be covered in search of suitable habitats. During one week, some individuals moved up to 742m (Table 3) but the average activity radius in this study had a mean ± SD of 270.78 ± 199.17m, which shows that there was a high variability among the adult individuals. On a yearly basis a frog could move up to a maximum total distance of 1,818m (Table 3). Studies in New York and Missouri (USA) showed that bullfrogs can move from 1,200 to 1,600m in one year (INGRAM & RANEY, 1943; WILLIS et al., 1956). This corresponds with our findings and suggests that the environmental circumstances within the valley of the Grote Nete are similar to the studied territories in the USA. These results show that dispersion of this invasive species within an ecosystem such as the valley of the Grote Nete can proceed very rapidly, which is confirmed by unpublished data on a public website (NATUURPUNT VZW, 2006). Adult and sub-adult bullfrogs also use rivers as a dispersion route (PETERSON et al., 2013) as confirmed by findings in our study where some individuals used and crossed the river in search of food and shelter. A female biased dispersion in bullfrogs is reported, where the males have a tendency to return to their birthplace with local reproductive resources (AUSTIN et al., 2003). A wider female dispersal can be expected because of the lack of parental care and the importance of mate choice in inbreeding avoidance and reproductive success (AUSTIN et al., 2003). A shorter multiple mark – recapture study in other pools in the valley of the Grote Nete found a substantial difference between adult male and female bullfrogs during the reproduction period (LOUETTE et al., 2013). However, these results could not be confirmed in our study, as no statistically significant difference was found between the movements made by males or females.

During the 24h-cycle trackings in the reproductive season most of the tracked frogs hardly changed position. Given that the larger ponds, where the animals were tracked, all had a very high abundance of larvae there must have been successful reproductions of the bullfrog. Our results suggest that reproductive movements are at short intervals during specific climatological circumstances. Daily tracking during this season would give better insight into the determining factors for reproductive migration. Overall weekly movements did occur during this period, which indicate that individuals moved back and forth to the reproduction sites. This migratory pattern was also observed in a pond in New Jersey (USA), where, during the breeding season, the movements from and to the pond were linked to environmental conditions such as rainfall and an elevation of the air temperature (RYAN, 1980). In our study, the movements towards the larger permanent ponds during the reproductive season were not simultaneous for all individuals that had a KDE50 in the alluvial forest. The period in which the males inhabited the permanent pond was longer than for the females. Three of the five tracked males in this study occupied a specific spot at the permanent ponds that also function as reproductive sites. The females shared more variations in positions during this period. These observations may confirm the fact that females arrive at asynchronous intervals at the breeding pond during the reproduction period because the duration of sexual activity for an individual female is extremely short, generally only one night (RANEY, 1940; EMLEN, 1976; RYAN, 1980). These findings suggest that males, as in the majority of amphibians, defend and hold their optimal reproduction site for a long time and females migrate to the pond for a shorter time to choose a mating partner and reproduce. This "resource defence polygyny" (Emlen & Oring, 1977) was also observed in a study at New Jersey, which showed that the males actively defended the oviposition sites in the ponds (Ryan, 1980). During a few nights in the mating season males form short-term calling aggregations, also called choruses. This spatial organisation reflects the social dominance of the males in the population. They aggregate for the purpose of attracting females, and females move actively to the choruses and select their mating partner (Emlen, 1976). EMLEM (1976) also stated that, during mating season, males are highly mobile and move from one aggregation to another. Moreover, as shown by RANEY (1940), the movements took place after sunset and during or after rainfall and were not correlated with foraging, egg-laying or temperature changes. A higher mobility of male adult bullfrogs could not be confirmed in our study in which males stayed at their specific spot during the reproduction period. A possible explanation for this phenomenon is that those male aggregations are very rare or unique in a specific pond and consequently so also are the movements towards it. The frequency or time of tracking adopted in this study did not record these brief displacements. GPS-telemetry would be a better option to track these movements in the future, but suitable GPS-transmitters were not available at the time of the study.

The analyses of the distances covered by the adult bullfrogs during the tracking period revealed a statistically positive correlation between the weight of the animal and the maximal distance covered during one displacement event. This suggests that heavy and consequently dominant animals tend to search more for an optimal habitat for reproduction, foraging, hibernation and shelter.

The present conservation plans and measures for SAC in the valley of the Grote Nete focus on rewetting and creating wet corridors between the different parts of the valley (Agentschap voor Natuur en Bos, 2012). Considering the results from this study, these plans will favour and enhance the dispersion of this invasive species and create more suitable habitats as well. The marsh habitat characteristics of this large ecosystem and the fact that the population is already widely spread (distance of 42km in the valley), impede an active control of the populations of the American bullfrog in this valley. Such active control methods include removing adults, sub-adults and especially larvae with fykes and are the only measures taken at the moment. Therefore, passive control systems are urgently needed in these conditions in order to reduce the high local impact of this invasive species on the native species. A possible strategy is the use of the 'Sterile-Male-Release'-technique (Patterson et al., 1968). This technique is a structural and sustainable method to eradicate or control large population of invasive exotic species. The release of a high number of sterile males of the invasive species is necessary to reduce the amount of successful fertilizations. In the male sea lamprey (*Petromyzon marinus* Linnaeus, 1758) the sterility does not affect the mating instinct and competitive behavior (Bergstedt & Twohey, 2007). The release of sex pheromones is not inhibited either, so ovulating females will still be attracted (Bergstedt & Twohey, 2007). A combination of removal of female sea lampreys by traps and the sterile male release resulted in an average population reduction of 64% over eight years in the Great Lakes region (US) (Bergstedt et al., 2003).

More research is needed into the development of sustainable, cost effective and labor-extensive techniques to control widely extended populations of bullfrogs or other invasive alien species. Trapping adults combined with functional sterility of male individuals, and their subsequent release, could provide a long-term solution to control these types of invasive populations.

CONCLUSIONS

The dispersion of the invasive American bullfrog proceeds very rapidly in a river ecosystem such as the valley of the Grote Nete (Belgium). This study showed a statistically significant correlation between the weight of the animal and the distance they covered in a single movement, suggesting that more dominant animals will disperse faster in the surrounding landscape. The tracked bullfrogs had a small core range habitat with high spot fidelity, which provided them with food and shelter in the shoreline of permanent ponds or the alluvial forest. An effective method is needed to control the wide dispersion of this invasive species in the valley of the Grote Nete to safeguard the local biodiversity in this SAC.

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Investigation of ancient DNA to enhance natural history museum collections: misidentification of smooth-coated otter (*Lutrogale perspicillata*) specimens across multiple museums

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ABSTRACT. Historical and modern natural museum collections are storehouses of extraordinary value for scientific research in a wide range of fields. Recent advances in molecular biotechnology (e.g., next generation genomics) have increased the range of collection material employable for DNA-based analyses to unprecedented levels. Nevertheless, the value of museum specimens strictly depends on reliability of data associated with them. We report on investigations of ancient DNA from specimens of smooth-coated otter (*Lutrogale perspicillata*, Mustelidae), the largest otter species living in Asia, in US and European mammal collections. Mitochondrial DNA Cytochrome-*b* gene sequencing proved that the studied specimens were not the expected taxon. Indeed, they actually belonged to three different species, namely the Asian small-clawed (*Aonyx cinereus*), Eurasian (*Lutra lutra*) and African clawless (*Aonyx capensis*) otters. This represents the first record of mustelid misidentification from museum collections. Detection of errors can be extremely difficult when based only on collectors' notes and data. Hence, we warn scientists involved in otter research about potential challenges when dealing with museum specimens. We recommend curators pursue a multidisciplinary approach, including DNA analyses, to accurately catalogue the resources under their management and uphold the value of biodiversity information.

KEY WORDS: error, genetic identity, mistaken cataloguing, mitochondrial DNA, specimen label

INTRODUCTION

Natural history museums first appeared in Europe during XVI century as cabinets of curious, artificial and natural items (*wunderkammer*) for nobles, dealers and travellers. Since then, they have progressively grown in relevance as authentic scientific collections, and support research in a wide range of fields, from systematics to ecology and evolutionary biology (Thomas, 1994; Weschler, 1995). In the 1980s, PCR-based techniques also allowed retrieval of molecular information from museum specimens. Rapidly, pioneering studies obtained the first DNA sequences from extinct taxa (Higuchi et al., 1984; Thomas et al., 1989), and collections began to play a role as potential storehouses of astonishing value for a huge array of scientific investigations (Gee, 1988; Graves & Braun, 1992).

The more refined the molecular-genetic techniques became, the more appealing were museum specimens, even in ways that the original collector had never imagined before (e.g., next generation genomics: Bi et al., 2013). Funding shortages to support wide-ranging and long-lasting sampling in the wild, socio-political instability of study areas, rarity and/or elusiveness of taxa (especially if at risk of extinction), and the need for ecological data series, were all factors that fuelled a growing interest in museum specimens (e.g., Suarez & Tsutsui, 2004; Wandeler et al., 2007; Lister

& Climate Change Research Group, 2011). Hence, DNA study of material in collections increased to unprecedented levels. If specimens are to be an essential tool in research, their value depends completely on reliability of data associated with them (Boessenkool et al., 2010). Therefore, errors (taxonomic identity, origin, gender, etc.) disclosed by recent DNA-based investigations have been vital findings for the management of museum biodiversity resources. However, these studies focused on mammals (*Panthera*: Barnett et al., 2007; *Bradypus*: de Moraes-Barros et al., 2011; rodents: Müller et al., 2013; Robins et al., 2014) and birds only (*Gallinula*: Lee & Griffiths, 2003; *Megadyptes*: Boessenkool et al., 2010; *Acrocephalus*: Koblik et al., 2011; *Leucocarbo*: Rawlence et al., 2014; *Francolinus*: Forcina et al., 2015).

Otters (Mustelidae, Carnivora) include 13 species living on all continents except Antarctica and Australasia. Despite some being diurnal, otters are elusive and cryptic in habit, and they can be difficult to observe in the wild. For this reason, most studies rely on non-invasive sampling methods (faeces: e.g., Lerone et al., 2014), road-killed (or died from other causes) individuals (e.g., Koepfli et al., 2008a) and museum frozen tissue collections (e.g., Koepfli et al., 2008b) to increase sample size obtained from wild-captured animals. This is the case also for the smooth-coated otter (*Lutrogale perspicillata*), the largest living Asian otter, whose distribution range encompasses socio-politically unstable and remote areas, as the species occurs in Iraq with an isolated population (Al-Sheikhly & Nader, 2013; Al-Sheikhly et al., 2015a, b), and from Pakistan across India to southern China, Indochina and extreme southeastern Asia (Java and Borneo). The species is listed as Vulnerable by IUCN and its patchy population has globally declined by 30% over the past 30 years, meaning that in some place otters are now locally extinct (Hwang & Larivière, 2005; Hussain et al., 2008; Yoxon & Yoxon, 2014). In addition, unlike the Eurasian (*Lutra lutra*) and the Asian small-clawed (*Aonyx cinereus*) otter, live individuals of *L. perspicillata* are only kept in *ex situ* institutions in low numbers. Therefore, specimens resident in museum collections can represent a highly valuable resource for conservation, ecological, biogeographical and evolutionary research.

In this paper, we report on investigation of ancient DNA from otters labelled as *L. perspicillata* and resident in mammal collections of US and European museums. We have proved that these specimens belong to three different species, two sympatric with the smooth-coated otter in the region where they were collected, and one living in the African continent. We emphasize the need for a multidisciplinary approach, including DNA analyses, to properly identify museum otters.

MATERIALS AND METHODS

Museum specimen sampling

We borrowed samples from five otter specimens resident in the mammal collections of the natural history museums of Chicago, Paris and Vienna (Table 1). Curators provided a tiny amount (< 5 mg) of dry skin from the skull cavity (e.g., turbinates). Alternatively, we acquired slivers of toe pad. All specimens were catalogued as *Lutrogale perspicillata*, a taxon included in the Appendix II of CITES. Samples were shipped to the Department of Biology of Pisa, registered (IT 027 code) as CITES exempt scientific institution.

DNA extraction, amplification and sequencing

DNA was extracted in a dedicated room free of any mammal DNA in the Anthropology building of the Department of Biology (Zoology-Anthropology Unit). Workflow was conducted in strict conformity to ancient DNA protocols throughout all steps, including physically isolated pre-PCR and post-PCR working areas and with *ad hoc* equipment. UV light and 10% bleach were routinely used to sterilize the

Table 1

Museum specimens investigated in this study. * = referred to as from either French Indochina (original label) or Vietnam (specimen box); ** = skin bought at the market of Kathmandu; ? = not determined.

Specimen label	Museum	Specimen code	Sex	Age	Region	Locality	Date	Sample	Genetic ID	GenBank
Lutrogale perspicillata	Field Museum of Natural History, Chicago, USA	FMNH 37890	♂	A few weeks	Laos PDR	Thateng (Plateau des Bolovens: Lat. 15°33’N, Long. 106°33’E)	25 Dec. 1931	Skin from skull	*Aonyx cinereus*	LT220225
Lutrogale perspicillata	Field Museum of Natural History, Chicago, USA	FMNH 37891	♀	A few weeks	Laos PDR	Thateng (Plateau des Bolovens: Lat. 15°33’N, Long. 106°33’E)	25 Dec. 1931	Skin from skull	*Aonyx cinereus*	LT220225
Lutrogale perspicillata	National Museum of Natural History, Paris, France	MNHN-ZM-MO 1883-1295	♂	Juvenile	Philippines	Puerto Princesa, Palawan Is.	1883	Toe pad	*Aonyx cinereus*	LT220226
Lutrogale perspicillata	National Museum of Natural History, Paris, France	MNHN-ZM-MO 1962-1646	♀	Adult	Indochina	Unknown*	1962	Skin from skull	*Aonyx capensis*	LT220227
Lutrogale perspicillata	Natural History Museum, Vienna, Austria	NMW 43414	?	?	Nepal	Kathmandu**	1978	Toe pad	*Lutra lutra*	LT220228

surfaces of benches and laboratory devices, and to get rid of any possible contaminant DNA. The reliability of each DNA extraction was monitored through two blank controls. A small amount (2 mg) of starting material was removed from each sample and minced, employing a sterile disposable razor blade (BBraun, Aesculap Division). DNA was isolated using the QIAamp DNA Micro Kit (Qiagen) in compliance with the manufacturer’s instructions, modified as follows when dealing with hard tissues: (i) incubation in a shaking water bath up to 48h; (ii) use of 4 µl of dithiothreitol (Fluka, 4 mg/ml) every 24h of incubation; (iii) twofold addition of proteinase K (Sigma Aldrich, 20 mg/ml); (iv) repeated freezing and thawing of the supernatant, as it separated out residual proteins and other substances that seemed to inhibit PCR (Pergams & Lacy, 2008). We amplified two overlapping 211 bp-long and 199 bp-long mitochondrial DNA (mtDNA) Cytochrome-*b* gene (Cyt-*b*, total length: 1,140 bp) fragments in two distinct PCR reactions using primers reported in Table 2. Final (fragment 1 + fragment 2) 307 bp-long sequence corresponded to the Cyt-*b* portion comprised between nucleotide (nt) position n. 602 and n. 908 (codon reading frame = 2). PCR reactions (50 µl) were prepared as follows: 1 µl of Ampli*Taq* Gold DNA Polymerase (1 U/µl, Applied Biosystems), 4 µl 25 mM $MgCl_2$ (Applied Biosystems), 5 µl of 10x PCR Gold buffer (Applied Biosystems), 5 µl 2.5 mM dNTP (Sigma Aldrich), 3 µl of each primer (1 µM), 1 µl of DNA template and 1 µl of 75 µM Bovine Serum Albumin (4 mg/ml, Sigma Aldrich) to prevent proteins from inhibiting PCR (Pääbo et al., 1988). We carried out PCRs in an Eppendorf Master Cycler Personal (v5332) including two blank controls to check for cross contaminations. Thermal profile was as follows: 10 min at 94°C; then, 70 cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s; final extension, 72°C for 10 min. We purified PCR products using the Genelute PCR Clean-up Kit (volume 40 µl; Sigma Aldrich), and we directly sequenced them twice on both DNA strands (BigDye® Terminator v3.1 Cycle Sequencing Kit, ABI 3730 DNA automated sequencer, Applied Biosystems) at Genechron (ENEA, Rome, Italy).

Genetic analyses

Chromas v2.01 (http://chromas-lite.software.informer.com/2.0) was used to read ABI electropherograms, whereas ClustalX v1.81 (Thompson et al., 1987) was used to align partial Cyt-*b* sequences with those downloaded from the National Center for Biotechnology

Table 2
Primers used for the amplification of the two mtDNA Cyt-*b* fragments of this study.

Primer	5'-3' sequence	PCR product
Fw_583	GTTCACCTCCTGTTTCTCC	211 bp-long Cyt-*b* (fragment 1)
Rev_794	GGTGTACTGAGCGGGTTGGC	211 bp-long Cyt-*b* (fragment 1)
Fw_727	GTACTATTCTCCCCAGACCT	199 bp-long Cyt-*b* (fragment 2)
Rev_926	GAGGTGTGTAGCAGTGGGACG	199 bp-long Cyt-*b* (fragment 2)

Information (GenBank) and dealing with 12 out of 13 known otter species. These sequences were obtained from Koepfli et al. (2008a) with the exception of *Lontra provocax* (southern river otter: Vianna et al., 2011). No GenBank record was available for *Aonyx congicus* (Congo clawless otter). We used Mega v5 (Tamura et al., 2011) to calculate nucleotide composition and transitions: transversions *ratio* (Ti/Tv). Comparative sequence analyses were carried out using BioEdit v5.0.9 (Hall, 1999) to compute nucleotide difference count matrix for the whole alignment and to identify polymorphic sites among *A. cinereus*, *A. capensis*, *L. lutra*, *L. perspicillata* and the investigated museum specimens. Then, we produced a Maximum Likelihood (ML) tree choosing *Pteronura brasiliensis* (giant otter) as outgroup according to the molecular phylogeny of Koepfli et al. (2008b). However, no attempt was made to reconstruct the evolutionary relationships within Lutrinae due to the constraints of using a short fragment from a single genetic marker. We carried out a robust heuristic tree reconstruction in order to assign sequences retrieved from museum specimens to GenBank otter records. Following Guindon et al. (2010), we used Smart Model Selection at PhyML (South of France Bioinformatic Platform, www.atgc-montpellier.fr) and we found that the TN93 (Tamura & Nei, 1993) + G (a shape parameter = 3.69, with six substitution rate categories) + I (proportion of invariable sites = 0.54) was the best evolutionary model fitting to our dataset according to both Akaike (= 2804.8) and Bayesian (= 2946.4) Information Criterion. We used these parameters to carry out an ML reconstruction using Nearest-Neighbour Interchanges to swap adjacent tree branches (with active topology/branch length improving options). Statistic support at each node was evaluated by bootstrapping percentage (BP, with 1,000 replicates: Felsenstein, 1985).

RESULTS

Electropherograms were identical with each other for each specimen analysed in the study. Overall, we found average unequal nucleotide composition typical of animal mtDNA: 29.1% of adenine, 22.1% of thymine, 36.0% of cytosine, and 12.8% of guanine. The number of Ti was 9.1 times higher than that of Tv, on average. We did not detect any internal stop codon/indels. Then, the real mtDNA nature of the five PCR products was assessed, and the potential occurrence of any nuclear sequence of mitochondrial origin (Numt: *sensu* Lopez et al., 1994) was ruled out.

None of the sequenced specimens turned out to be *L. perspicillata* as was expected according to their labels, three (FMNH 37890-1 and MNHN-ZM-MO 1883-1295) being assigned to *Aonyx cinereus*, one (MNHN-ZM-MO 1962-1646) to *A. capensis* and one (NMW 43414) to *Lutra lutra* (Table 1). When the average number of nucleotide differences among otter species was taken into account, we found that it ranged between 7 (over 307 nt, 2.3%: *L. provocax vs*. *Lontra felina*) and 71 (over 307 nt, 23.1%: *Pteronura brasiliensis vs*. *Hydrictis maculicollis*) (Table 3). In particular, FMNH 37890 and FMNH 37891 sequences were 100% identical to *A. cinereus* AF057119 GenBank entry, while MNHN-ZM-MO 1883-1295 diverged from the latter by two nucleotide substitutions (= 99.3% of identity); MNHN-ZM-MO 1962-1646 and NMW 43414 were 100% identical to *A. capensis* AF057118 and *L. lutra*

Table 3

Nucleotide difference count matrix as inferred from aligned 307 bp-long mtDNA Cyt-*b* sequences. Legend: Lfel, *Lontra felina* (marine otter); Lpro, *Lontra provocax* (southern river otter); Llon, *Lontra longicaudis* (Neotropical otter); Lcan, *Lontra candensis* (North American river otter); Llut, *Lutra lutra* (Eurasian otter); 43414, NMW 43414 specimen; Lsum, *Lutra sumatrana* (hairy-nosed otter); Acin, *Aonyx cinereus* (Asian small-clawed otter); 37890, FMNH 37890 specimen; 37891, FMNH 37891 specimen; 1883, MNHN-ZM-MO 1883-1295 specimen; Lper, *Lutrogale perspicillata* (smooth-coated otter); Acap, *Aonyx capensis* (African clawless otter); 1962, MNHN-ZM-MO 1962-1646 specimen; Hmac, *Hydrictis maculicollis* (spotted-necked otter); Elut, *Enhydra lutris* (sea otter); Pbra, *Pteronura brasiliensis* (giant otter). GenBank code of each otter sequence used in this matrix is reported in Fig. 1 and Table 1.

	Lfel	Lpro	Llon	Lcan	Llut	43414	Lsum	Acin	37890	37891	1883	Lper	Acap	1962	Hmac	Elut	Pbra
Lfel	-																
Lpro	7	-															
Llon	19	12	-														
Lcan	31	24	28	-													
Llut	53	50	52	50	-												
43414	53	50	52	50	0	-											
Lsum	56	51	47	47	26	26	-										
Acin	48	43	42	45	36	36	30	-									
37890	48	43	42	45	36	36	30	0	-								
37891	48	43	42	45	36	36	30	0	0	-							
1883	48	43	43	47	36	36	30	2	2	2	-						
Lper	52	47	47	49	30	30	29	25	25	25	25	-					
Acap	58	55	53	50	33	33	26	32	32	32	34	29	-				
1962	58	55	53	50	33	33	26	32	32	32	34	29	0	-			
Hmac	59	54	56	54	45	45	43	47	47	47	49	50	45	45	-		
Elut	59	55	54	54	41	41	38	45	45	45	47	41	35	35	51	-	
Pbra	66	63	57	57	50	50	56	57	57	57	59	59	56	56	71	57	-

AF057124, respectively. Polymorphic sites for *A. cinereus*, *L. perspicillata*, *A. capensis*, *L. lutra* and museum specimens were included in Table 4. In agreement with what is reported above, ML reconstruction (Fig. 1) assigned specimens FMNH 378901-1 and MNHN-ZM-MO 1883-1295 to *A. cinereus* (BP = 95%), and MNHN-ZM-MO 1962-1646 and NMW 43414 to *A. capensis* and *L. lutra*, respectively (BP = 100%, both clusters).

DISCUSSION

The very large majority of museum specimens are correctly classified and catalogued. However, a small percentage includes various types of misinformation. Far from wanting to suggest that museum collections are somehow untrustworthy, we have reported some examples of smooth-coated otter misidentification in order to avoid perpetuation of errors and provide curators with correct information to enhance the value of their collection. Likewise, we warn scientists involved in otter research about such potential trouble. In this study, mtDNA Cyt-*b* gene sequencing indicated that five museum specimens recorded as *Lutrogale perspicillata* were incorrectly identified, as they belonged instead to three different species such as the Asian small-clawed (*A. cinereus*), Eurasian (*L. lutra*) and African clawless (*A. capensis*) otter. To the very best of our knowledge, these results represent the first record of mustelid misidentification from museum collection.

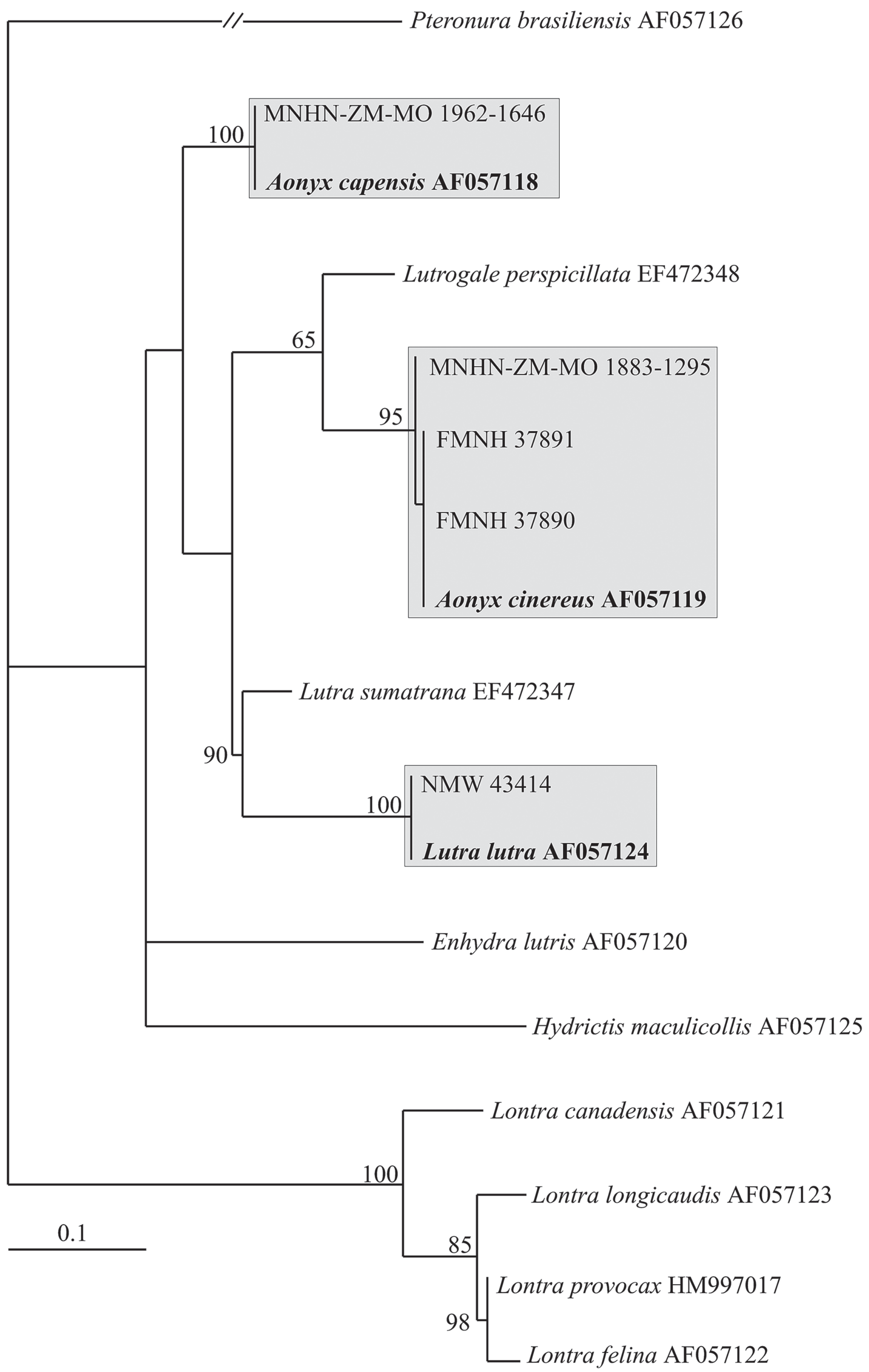

Fig. 1. – Maximum Likelihood tree reconstruction as obtained with PhyML using 307 bp-long mtDNA Cyt-*b* sequences of this study. Statistic support (bootstrapping percentage) is reported above each node when >50%. Scale bar is proportional to the number of substitutions per site. GenBank sequences were all obtained from Koepfli et al. (2008a) with the exception of *L. provocax* (Vianna et al., 2011). No genetic record was available for *A. congicus*. For detail on each species see Yoxon &Yoxon (2014).

Table 4
Polymorphic sites for *A. cinereus*, *L. perspicillata*, *A. capensis*, *L. lutra* and investigated museum specimens from aligned sequences. Dots refer to nucleotides that are identical in state. Nucleotide position number based on position within the alignment (1-307). Legend: 37890, FMNH 37890 specimen; 37891, FMNH 37891 specimen; Acin, *Aonyx cinereus* (GenBank code: AF057119); 1883, MNHN-ZM-MO 1883-1295 specimen; Lper, *Lutrogale perspicillata* (GenBank code: EF472348); Acap, *Aonyx capensis* (GenBank code: AF057118); 1962, MNHN-ZM-MO 1962-1646 specimen; Llut, *Lutra lutra* (GenBank code: AF057124); 43414, NMW 43414 specimen.

	1	3	3	5	6	8	8	8	9	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	2	9	9	2	3	4	9	2	9	0	0	0	0	0	1	1	2	2	2	2	4	4	5	7	7	7	8
											0	1	4	5	6	1	6	1	2	5	8	6	7	8	0	3	6	2
37890	C	T	G	C	T	T	G	A	C	C	T	C	A	A	T	A	A	C	C	G	G	A	C	A	T	C	T	A
37891	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Acin	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1883	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lper	.	.	.	.	C	.	A	G	.	.	.	T	G	G	.	G	.	.	.	A	A	.	.	T	C	.	C	G
Acap	A	C	.	.	.	C	A	G	.	T	C	.	.	G	C	G	.	T	A	.	A	.	.	.	C	T	C	.
1962	A	C	.	.	.	C	A	G	.	T	C	.	.	G	C	G	.	T	A	.	A	.	.	.	C	T	C	.
Llut	A	.	A	T	C	C	A	.	A	.	.	.	.	G	C	.	T	T	A	A	A	G	T	.	.	T	C	G
43414	A	.	A	T	C	C	A	.	A	.	.	.	.	G	C	.	T	T	A	A	A	G	T	.	.	T	C	G

	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3
	8	9	9	0	0	1	1	2	2	2	3	3	3	4	4	5	6	6	7	7	8	8	8	9	0	0	0	0
	7	4	7	6	9	2	5	1	4	7	0	3	9	2	8	4	3	9	3	5	1	2	5	0	0	2	5	6
37890	G	A	C	G	T	G	G	C	A	C	A	C	T	G	T	C	G	A	C	A	A	A	C	T	C	A	C	G
37891	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Acin	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1883	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.
Lper	.	.	T	.	.	A	A	.	.	.	.	.	.	A	C	.	A	G	T	G	.	.	T	C	.	.	.	A
Acap	.	.	.	A	C	A	A	.	G	T	.	T	C	.	C	.	A	.	.	.	G	.	T	C	T	G	.	A
1962	.	.	.	A	C	A	A	.	G	T	.	T	C	.	C	.	A	.	.	.	G	.	T	C	T	G	.	A
Llut	A	.	.	A	.	A	A	T	.	.	G	.	C	A	C	T	T	.	T	G	.	G	.	C	.	.	T	A
43414	A	.	.	A	.	A	A	T	.	.	G	.	C	A	C	T	T	.	T	G	.	G	.	C	.	.	T	A

Museum specimens from Chicago (Table 1) were wild otters caught by the same collector on the same day and locality in Laos. They were a few-week-old individuals; hence, their identification could have hardly been more than an hypothesis (L Heaney, pers. com. to F Barbanera, 2014). Likely, these otters were litter mates, as supported by the fact they hold the same *A. cinereus* mtDNA Cyt-*b* haplotype (see Results). Samples from two additional wild-caught juvenile otters collected during the same expedition in the same area were borrowed from the Chicago museum. They proved to be genuine *L. perspicillata* otters when their mtDNA was sequenced in Pisa (MORETTI et al., in preparation). Therefore, on the one hand, sympatric occurrence of *A. cinereus* and *L. perspicillata* in Laos has been confirmed. On the other hand, we suggest that young representatives of these species cannot be reliably identified on a morphological basis (skull). The two species, indeed, show very similar brain structure (RADINSKY, 1968; WILLEMSEN, 1980), which is in agreement with their strict phylogenetic relationships proved by KOEPFLI et al. (2008a), who eventually suggested including them both in the genus *Amblonyx* (RAFINESQUE, 1832).

Museum specimens from Paris (Table 1) led to very different outcomes. Specimen MNHN-ZM-MO 1883-1295 was collected on the island of Palawan (Philippines), less than 300 km away from Malaysian Borneo (Sabah Province). The latter represents the easternmost edge of current smooth-coated otter distribution range (YOXON & YOXON, 2014). Hence, we were particularly interested in this specimen to assess if *L. perspicillata* formerly occurred in the

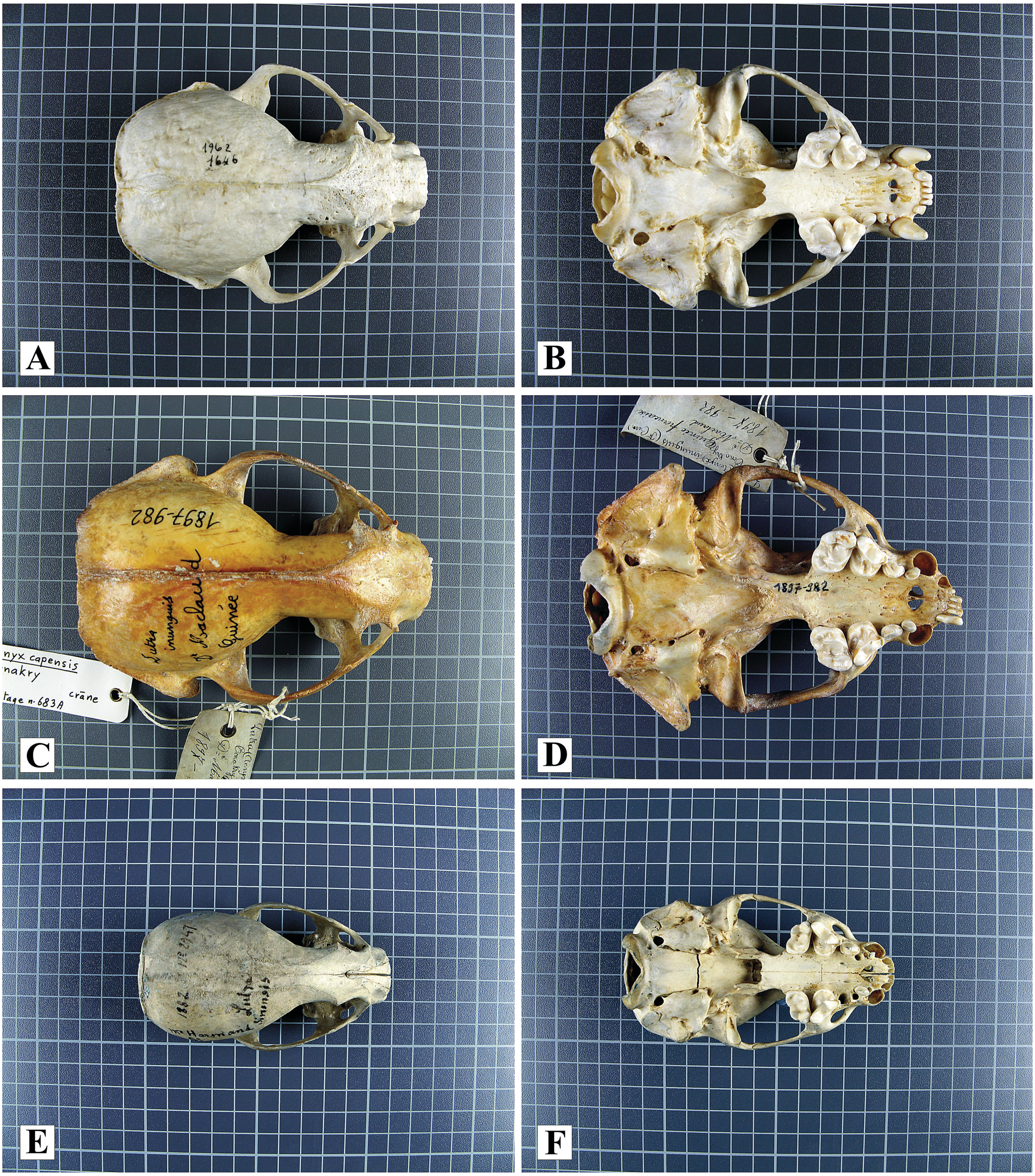

Fig. 2. – Dorsal (left) and ventral (right) skull views of: A–B. Misidentified MNHN-ZM-MO 1962-1646 specimen (unknown locality, Indochina). C–D. *Aonyx capensis* CG 1897-982 (Conakry, Guinea). E–F. *Lutrogale perspicillata* CG 1882-2947 (unknown locality, Thailand). All specimens are resident in the mammal collection of the National Museum of Natural History of Paris. Photos: courtesy of Geraldine Veron (researcher and curator of mammal collection). Scale: 1 cm.

western Philippine islands as well. By using a sample obtained from a study skin, we found that MNHN-ZM-MO 1883-1295 belonged to the species *A. cinereus*. Later, we became aware that the skull of the same specimen, preserved separately from the skin, had been catalogued as *A. cinereus*, thus confirming the reliability of the genetic result (G Veron, pers. com. to F Barbanera, 2014). Furthermore, researchers in the Philippines confirmed the absence of *L. perspicillata* in Palawan, the Asian small-clawed being the only otter recorded so far (LSG Castro & DAP Fernandez, pers. com. to F Barbanera, 2015).

As far as the second specimen (MNHN-ZM-MO 1962-1646) from Paris is concerned, we found that it was not *L. perspicillata* from Indochina but an African clawless otter (*A. capensis*). This result came as a big surprise, but it was confirmed following a morphological comparison between the skull of the specimen in point (Fig. 2: A, B) and those of real *A. capensis* and *L. perspicillata* kept in the same collection (Fig. 2: C to F). To summarize, *A. capensis* (Fig. 2: C, D) has a broader and more rounded brain case than *L. perspicillata* (Fig. 2: E, F). The latter is evenly ovoid and much deeper than wide, with a high rostrum (blunt in *A. capensis*). *Aonyx capensis* has wider orbits and shorter zygomata (with wide and prominent posterior temporal process) than *L. perspicillata*. The infraorbital foramen is rounded in *A. capensis* and kidney-shaped in *L. perspicillata*, and deeper in the first than in the second. The anterior palatine foramen is wide and subtriangular in shape in *A. capensis* while it is small and rounded in *L. perspicillata*; the sagittal crest is placed upward in *A. capensis* while it is low in *L. perspicillata* (cf. Harrison & Bates, 1991).

NMW 43414 otter skin from Vienna, the most recently collected specimen (1978) of this study (Table 1), was bought at the market of Kathmandu, Nepal. Regrettably, no further information was available. We assigned this specimen to *L. lutra*. Smooth-coated differs from Eurasian otter in having a more massive head and heavier teeth, shorter and smoother fur, sleek appearance, and dorsoventrally rather than circular flattened tail tip (Hwang & Larivière, 2005). However, otter identification can be difficult, especially when based only on old and/or not well-preserved dry skin. As reported by Al-Sheikhly & Nader (2013), who made morphometric analyses of skins from dead otters in order to prove the persistence of *L. p. maxwelli* subspecies in Iraq, loss of pelage colour is common in old specimens and creates a similar appearance to Eurasian and smooth-coated otters. Nevertheless, skin of both species can be reliably identified by inspecting rhinarium and eyehole position. In the smooth-coated otter, the upper border of the rhinarium shows a well-defined hairline, which is much straighter than in the Eurasian otter. In the latter, it appears as convex. Furthermore, in the smooth-coated otter the eyehole is placed more anteriorly and considerably lower down in the face when compared to the Eurasian otter (Harrison & Bates, 1991).

As comprehensively discussed by Rasmussen & Prîj-Jones (2003), there is a wide range of ways through which misinformation can spread across museum collections, spanning from casual errors and careless labelling to commercial imprecision, incompetence (inadequate training and/or supervision of collectors), inappropriate curatorial techniques, problems in deciphering and interpreting data, and even fraud. Regrettably, detecting such errors can be extremely challenging. As interestingly noted by Boessenkool et al. (2010), investigation on doubtful specimens is usually undertaken when they are from a suspicious collector, form outliers with respect to the natural distribution range of a given taxon, or show an unconvincing collection date (e.g., after a species was reported to be extinct). With reference to our study, we do not have enough information to disentangle how mistakes occurred. Chicago specimens were baby otters, and morphological approach for their identification proved to be unreliable. Despite concern that MNHN-ZM-MO 1883-1295 from the Philippines could be a suspicious outlier on the basis of present-day distribution range of

L. perspicillata, the error was detected only after DNA investigation, which prevented its wrong incorporation into any future report on historical diversity patterns of the species. Possibly, the separation of skin and skeletal remains in the collection did not help earlier disclosure of such erroneous labelling. Finally, wrong identification of the second specimen from Paris and of that from Vienna was possibly due to incompetence of collectors and subsequent carelessness of curators. Indeed, as reported above, dissimilarity between *L. perspicillata* and *L. lutra*/*A. capensis* should have been acknowledged. NMW 43414 wrong labelling could have originated also from dealers, as they usually prioritise profit over the correct identity of the item (Rasmussen & Prîj-Jones, 2003). Nevertheless, misidentification was perpetuated when the presently retired curators in Vienna determined the NMW 43414 skin as *Lutrogale*.

Historical material is a limited resource and museum sampling for molecular DNA use is a destructive procedure. Criteria for the approval of loans have been discussed since the early 1990s (e.g., Pääbo et al., 1992). Revealing errors in museum specimens can be very challenging when the investigation is mostly based on collectors' notes, data and preparatory techniques (Boessenkool et al., 2010). The ever-increasing results provided by the use of biotechnological methods suggest that the information obtained through DNA analyses may add huge value to a given specimen and/or museum collection. We encourage curators to pursue a multidisciplinary approach, including DNA analyses, to properly archive the resources of biodiversity under their management, and researchers to endorse full responsibility justifying their need for destructive sampling.

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Lethal and sublethal effects of spirotetramat and abamectin on predatory beetles (*Menochilus sexmaculatus*) via prey (*Agonoscena pistaciae*) exposure, important for integrated pest management in pistachio orchards

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ABSTRACT. *Menochilus sexmaculatus* Fabricius (Coleoptera: Coccinellidae) is an important biological control agent in pistachio orchards, especially against *Agonoscena pistaciae* Burckhardt and Lauterer (Hemiptera: Psyllidae), which is the most damaging pest of pistachio. In this project we exposed *M. sexmaculatus* adults to two important commonly-used insecticides through feeding on treated prey (*A. pistaciae*) to evaluate the side-effects on this predator. We tested spirotetramat, which belongs to the keto-enol group inhibiting lipid biosynthesis in insects, at 2/1, 1/1 and 1/2 of the maximum field recommended concentration (MFRC), and abamectin, which is a mixture of avermectins and a natural fermentation product of the bacterium *Streptomyces avermitilis*, at 1/1, 1/2, 1/4, 1/8 and 1/16 of its MFRC. Spirotetramat did not affect adult survival of *M. sexmaculatus* at all three concentrations when ingested via treated prey, while in marked contrast abamectin caused 100% adult mortality of *M. sexmaculatus* when ingested via treated prey at 1/1, 1/2, 1/4 and 1/8 of the MFRC. At sublethal levels, spirotetramat reduced total and daily fecundity of *M. sexmaculatus* at all three concentrations tested, but did not affect egg hatching at 1/1 and 1/2 of the MFRC. Moreover, prey consumption was decreased when beetles were exposed to the prey treated with spirotetramat at 1/1 and 2/1 of the MFRC concentrations. With abamectin, even at 1/16 of the MFRC, total fecundity, daily fecundity and prey consumption of *M. sexmaculatus* adults were significantly affected. In conclusion, no acute toxicity was observed on *M. sexmaculatus* by ingestion of prey treated with spirotetramat, although reproduction parameters and prey consumption were affected at MFRC and lower concentrations. In marked contrast, abamectin was notably very harmful at its MFRC and also at lower concentrations. This research highlighted the importance of toxicity risk assessments, including lethal and sublethal effects, to obtain a more accurate estimation of the compatibility of insecticides in current integrated pest management (IPM) programs.

KEY WORDS: abamectin*, Agonoscena pistaciae, Menochilus sexmaculatus*, predators, spirotetramat, sublethal effects

INTRODUCTION

The common pistachio psyllid, *Agonoscena pistaciae* Burckhardt and Lauterer (Hemiptera: Psyllidae), is the most damaging pest of pistachio (*Pistacia vera*) in Iran (Mehrnejad, 2001). Chemical control is a common method in management of this pest. However, continued use of chemical insecticides has made *A. pistaciae* resistant to the insecticides that are currently used. Additionally, some of these pesticides adversely affect the biological control agents that are currently used to control the psyllid and this in turn results in new pest outbreaks (Mehrnejad, 2003). Coccinellids usually play a key role in integrated pest management (IPM) programs in several agroecosystems (Jacas & Urbaneja, 2010). The ladybeetle *Menochilus sexmaculatus* Fabricius (Coleoptera: Coccinellidae) is an important biological control agent in various parts of the world, especially in East Asia. Its activities as a predator of different species of aphids and psyllids have been reported, but because of the physiological similarity between the pest and the natural enemy, pesticides often cause mortality in both groups of organisms (CROFT, 1990). In addition to the direct lethal effects of pesticides, estimated on the basis of mortality rate, sublethal effects of pesticides can also strongly influence physiology and behavior, affecting the population build-up and predation capacities of natural enemies (JOHNSON & TABASHNIK, 1999). Spirotetramat is a new systemic and persistent foliar insecticide and a tetramic acid derivative with a novel mode of action, interfering with lipid biosynthesis. In 2011, the use of this pesticide was authorized in several European countries (i.e., United Kingdom, Belgium and Switzerland) in different crops, such as brassicas and lettuce to control sucking pests (BAYER CROP SCIENCE, 2012). Despite its use in several areas on different crops, information on side-effects of spirotetramat on coccinellids is still scarce (BRUCK et al., 2009). The few studies available have categorized this lipid biosynthesis inhibitor as harmless to other natural enemies, such as the predators *Episyrphus balteatus* (de Geer) (Diptera: Syrphidae) and *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (SCHNORBACH et al., 2008; MOENS et al., 2011), *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) (PLANES et al., 2013). Abamectin is a naturally derived acaricide/insecticide isolated from fermentation of the soil microorganism *Streptomyces avermitilis*. Susceptibility to abamectin has been shown for several ladybeetle species (Coleoptera: Coccinellidae), including *Harmonia axyridis* Pallas, *Cryptolaemus* sp., *Cycloneda sanguinea* Linnaeus, and *Stethorus punctum* (LeConte) larvae and adults (MICHAUD, 2002; YOUAN, 2003; SEAL et al., 2006).

The assessment of pesticides in the registration procedure and for compatibility in IPM programs usually begins with a calculation of their acute toxicity, which provides essential information on the risk they may pose against natural enemies (CANDOLFI et al., 2001). However, researchers have documented the importance of sublethal effects of insecticides on different biological parameters of predators and parasitoids (BIONDI et al., 2012). Recently, studies have been done on biological and behavioral influences of insecticides on natural enemies (SCHNEIDER et al., 2008; DELPUECH et al., 2012; WRINN et al., 2012; ZOTTI et al., 2013). Therefore, the objective of this study was to assess the lethal and sublethal side-effects of spirotetramat and abamectin, two insecticides that are commonly used in pistachio orchards of Iran, on the adults of *M. sexmaculatus*. We exposed the predator to the insecticides at several concentrations relative to their maximum concentrations as recommended for use in the field (MFRC), via feeding on treated prey. These data are important for an adequate environmental risk assessment based on lethal and sublethal effects. The results will provide a measure of the compatibility of spirotetramat and abamectin in the current IPM programs with *M. sexmaculatus*, which is a good natural enemy against *A. pistaciae*, the key pest of pistachio in Iran.

MATERIALS AND METHODS

Insect

All developmental stages of *M. sexmaculatus* were reared in air-ventilated plastic boxes (20 × 25 ×10 cm) at 25 ± 2ºC, 65 ± 5% RH and a photoperiod of 16:8 (L:D) as previously described (FARHADI et al., 2011). In essence, the ladybeetles were provided with fresh pistachio leaves containing *A. pistaciae* as food. To prevent fungal growth, leaves were changed daily and the leaves containing beetle eggs were separated and transferred to Petri dishes. Due to cannibalistic behavior of the predatory larvae after hatching, individual larvae were transferred to separate Petri dishes. After two generations of breeding and feeding on *A. pistaciae* for adaptation, adults of *M. sexmaculatus* were used for experiments.

Insecticides

Commercial formulations of spirotetramat (Movento, 10% SC, 100 g of a.i./liter, Bayer Crop Science) and abamectin (Vertimec 1.8% EC, 18 g of a.i. per liter, Agriphar, Belgium) were used. Spirotetramat was investigated at a dilution series of 2/1, 1/1 and 1/2 of its MFRC, corresponding to 100, 50 and 25 mg/L, and abamectin at a dilution series of 1/1, 1/2, 1/4, 1/8 and 1/16 of its MFRC, corresponding to 9, 4.5, 2.25, 1.12 and 0.56 mg/L, respectively.

Lethal effects of spirotetramat and abamectin on survival of adults of *M. sexmaculatus*

To assess the lethal effect of spirotetramat and abamectin on *M. sexmaculatus* adults, pairs of one female and one male adult were randomly selected after we observed the first mating, and these pairs were placed in individual Petri dishes of 90 mm diameter. Subsequently, adult beetles were offered prey that had been treated by a dipping method (Farhadi et al., 2011) with one or the other insecticide (for spirotetramat at 2/1, 1/1 and 1/2 of its MFRC, and for abamectin at 1/1, 1/2, 1/4, 1/8 and 1/16 of its MFRC). In brief, the pistachio leaves containing *A. pistaciae* psyllids were dipped for 5 s in one of the different concentrations of pesticide. Then, after 0.5 hour of drying at room temperature, 150 live psyllid nymphs of the 4^{th}- or 5^{th}-instar were collected from the leaves and placed on non-infected leaf discs together with one pair of *M. sexmaculatus* predatory adults. We checked mortality after 24, 48 and 72 h. In the control groups, the leaves were dipped in distilled water. Each chemical treatment level involved three replicates of 10 pairs of adult ladybeetles.

Sublethal effects of spirotetramat and abamectin on consumption of *A. pistaciae* by *M. sexmaculatus*

In this experiment, pairs of one female and one male adult were randomly selected as the first mating was observed, and each pair was placed in a separate Petri dish of 90 mm diameter. Subsequently, the adult beetles were fed with insecticide-treated prey using the same leaf disc dipping method as described above. Also, as above, 150 living psyllid nymphs of the 4^{th}- or 5^{th}-instar were collected from the leaves and placed on non-infected leaf discs together with one pair of *M. sexmaculatus* adults. Each insecticide treatment level involved three replicates, each consisting of 10 pairs of adult beetles. In a separate experiment 10 males were each offered 70 treated psyllid nymphs of the 4^{th}- or 5^{th}-instar, and studied under identical conditions to determine the number of prey that were consumed by *M. sexmaculatus* males. To calculate the daily numbers of prey eaten by females, the average number of prey eaten by males was subtracted from the average number of prey eaten by pairs (Farhadi et al., 2011). To obtain the daily feeding rate, any uneaten nymphs were collected each day and replaced by freshly treated nymphs. The prey consumption by predators was recorded daily during a period of two weeks. In the control groups, the leaves were dipped in distilled water.

Sublethal effects of spirotetramat and abamectin on reproduction of *M. sexmaculatus*

M. sexmaculatus adults were exposed to the two insecticides via ingestion of treated prey as described previously (Farhadi et al., 2011). In brief, for each treatment, ten pairs (male and female) were selected after first mating and each pair was placed in a separate Petri dish to determine reproductive parameters. Adults were fed daily with freshly treated prey for two weeks. Egg hatching, total fecundity, daily fecundity, pre-oviposition period and survival were recorded on a daily basis during a period of 30 days. In the control groups, the leaves were dipped in distilled water.

TABLE 1

Lethal effect (% mortality) of spirotetramat and abamectin at different dilutions of their respective MFRC on the survival of adults of *M. sexmaculatus* when fed on treated prey (*A. pistaciae*) for 24, 48 and 72 h.

	Mortality (%)		
	24h	48h	72h
Spirotetramat[1]			
2/1 MFRC	0±0[a]	0±0[a]	0±0[a]
1/1 MFRC	0±0[a]	0±0[a]	0±0[a]
1/2 MFRC	0±0[a]	0±0[a]	0±0[a]
Abamectin[2]			
1/1 MFRC	100±0[c]		
1/2 MFRC	100±0[c]		
1/4 MFRC	95±5[c]	100±0[c]	
1/8 MFRC	0±0[a]	65±5[b]	100±0[c]
1/16 MFRC	0±0[a]	0±0[a]	0±0[a]

Data are expressed as mean percent mortality ± SE. Mortality was 0±0% in the control groups. Percentages followed by different letters are significantly different (P<0.05, Tukey HSD test).

[1]MFRC for spirotetramat=50 mg/ml

[2]MFRC for abamectin=9 mg/ml

Data analysis

Data were analyzed using SPSS software and Excel 2010. In addition, the lethal effects of the tested insecticides were categorized into four groups based on the guidelines of the International Organisation for Biological and Integrated Control (IOBC) where the toxicity is related to the life parameter reduction, expressed as percentage: harmless (< 30%), slightly harmful (30-79%) and moderately harmful (80-99%) to harmful > 99% (STERK et al., 1999). Biological parameters including egg-hatching, total fecundity, daily oviposition, pre-oviposition period and survival were tested for normality, and means with significant differences were separated by analysis of variance (ANOVA) followed by a Tukey HSD test at P < 0.05. Mortality score was corrected using Abbott's formula (ABBOTT, 1925).

RESULTS

Lethal effects of spirotetramat and abamectin on survival of adults of *M. sexmaculatus*

As shown in Tables 1 and 2, spirotetramat at the different concentrations tested (2/1, 1/1 and 1/2 of its MFRC) posed no negative effects on the survival of adults fed treated prey during the whole experiment of 72 h (harmless).

In marked contrast, abamectin at 1/1 and 1/2 of its MFRC killed all adults within the first 24 h of exposure to treated prey (harmful), and even at the 1/4 and 1/8 of the MFRC concentration there was 100% mortality at 48 h and 72 h (harmful), respectively. With the lowest concentration tested, 1/16 of its MFRC, there was no mortality at 72 h (harmless).

Sublethal effects of spirotetramat and abamectin on consumption of *A. pistaciae* by *M. sexmaculatus*

The female adults of *M. sexmaculatus* that fed on prey treated with spirotetramat showed sublethal effects, consuming significantly fewer psyllids per day at 2/1 (30% fewer) and 1/1 (21% fewer) of the MFRC (P<0.001) (Table 3). At 1/2 of its MFRC, spirotetramat caused only a 6% reduction in psyllid consumption, which was non-significant.

For abamectin, we tested for potential sublethal effects using of 1/16 of its MFRC, because all the higher concentrations killed all adults

TABLE 2

Toxicity categories, using IOBC guidelines, of spirotetramat and abamectin at different dilutions of their respective MFRC, based on survival of *M. sexmaculatus* adults when fed on treated prey (*A. pistaciae*) for 24, 48 and 72 h.

	Toxicity levels		
	24 h	48 h	72 h
Spirotetramat [1]			
2/1 MFRC	harmless	harmless	harmless
1/1 MFRC	harmless	harmless	harmless
1/2 MFRC	harmless	harmless	harmless
Abamectin [2]			
1/1 MFRC	harmful		
1/2 MFRC	harmful		
1/4 MFRC	moderately harmful	harmful	
1/8 MFRC	harmless	slightly harmful	harmful
1/16 MFRC	harmless	harmless	harmless

[1] MFRC for spirotetramat=50 mg/ml

[2] MFRC for abamectin=9 mg/ml

within 72 h (Table 1). Feeding was also reduced by about 32% with exposure to this very low concentration of abamectin as compared to the control (P<0.05) (Table 3).

Sublethal effects of spirotetramat and abamectin on reproduction of *M. sexmaculatus*

As shown in Table 4, adult survival was 100% during the experiment of 30 days with spirotetramat at the three concentrations tested. However, reproduction parameters were affected. The strongest effect was seen in daily fecundity and total reproduction (i.e., total number of eggs during the period of 30 days of the experiment) (both P<0.001). Particularly with the highest concentration tested (2/1 of the MFRC), daily fecundity was reduced by 64% compared to the control, and subsequently this resulted in a reduction of the total fecundity (by 66%). In addition, these reproductive effects from 2/1 of the MFRC of spirotetramat were combined with an increase of 55% in the pre-oviposition period (i.e., time needed for first oviposition) (P<0.001) (Table 4). In addition, for the eggs deposited by the adults that fed on prey treated with 2/1 of the MFRC of spirotetramat, hatching of 1st-instar nymphs was reduced slightly (10%) but the effect was significant (P=0.015).

For abamectin at 1/16 of its MFRC, the effects on reproduction were also significant (Table 4). There was a loss of adult survival of 20%, total fecundity was reduced by 35% and daily fecundity by 20% (both P<0.001), while pre-oviposition period and egg-hatching were not affected (both P>0.05).

DISCUSSION

Spirotetramat is a relatively new compound mostly targeting hemipterans. It is a keto-enol derivative of tetronic acid, acting mainly by inhibiting lipogenesis following ingestion. The U.S. Environmental Protection Agency (US EPA, 2008) noted that the basic risk to bees seems to be low based on acute oral and contact experiments with honey bees. However, they reported that brood feeding trials with bees, and acute toxicity and contact studies with other non-target insects (e.g. parasitoid wasps and predatory mites) conducted at less than the maximum application rate, suggested a risk for mortality in adults and pupae, substantial agitation of brood development, and early brood

TABLE 3

Sublethal effect of spirotetramat and abamectin at different dilutions of their respective MFRC on daily feeding rate of *M. sexmaculatus* females fed on treated prey (*A. pistaciae*) for 2 weeks, compared to the control (water).

	Spirotetramat [1]			Abamectin [2]	Control
	2/1 MFRC	1/1 MFRC	1/2 MFRC	1/16 MFRC	
Number of prey ingested	64.9±2.5[bc]	71.6±1.3[b]	85.6± 1.3 [a]	62.2±2.4[c]	90.8 ±0.8[a]

Data are expressed as means±SE. Means followed by different letters are significantly different (P<0.05, Tukey HSD test).
[1]MFRC for spirotetramat=50 mg/ml
[2]MFRC for abamectin=9 mg/ml

termination. Currently, spirotetramat can be classified as practically non-toxic to birds and mammals on an acute basis, and practically non-toxic to honeybees based on acute oral and contact studies. Our results demonstrated no acute mortality to ladybeetles upon exposure to 2/1, 1/1 and 1/2 of the MFRC. However, we found that spirotetramat reduced consumption of prey at 1/1 and 1/2 of the MFRC. (Spirotetramat is not a fast acting insecticide, and all prey remained alive and available for the ladybeetles to catch.) Similar results have been observed in other studies where spirotetramat reduced consumption of prey by *Galendromus occidentalis* (Acari: Phytoseiidae) (Beers & Schmidt, 2014). Our studies, at higher dosages of 2/1 MFRC, found that spirotetramat had a low but significant effect on egg hatching (10% reduction) but substantial effects on fecundity and time to first oviposition. Significant effects were even seen on fecundity from spirotetramat application at 1/2 of the MFRC. These greater effects on the adult than on its eggs suggest that timing of application of this insecticide may be important in minimizing harm to this predator. Consequently, we believe that similar studies should be undertaken using more field-related conditions before spirotetramat can be recommended for use in IPM programs. Recently, Beers & Schmidt (2014) reported that spirotetramat decreased egg hatching and fecundity of *G. occidentalis* at 1/10, 1/1 and 2/1 of its MFRC, but Planes et al., 2013 did not find such effects on *C. montrouzieri* by similar topical treatment and ingestion. It appears that the side-effects of this insecticide may depend on the insect species. Similar to our results, several recent studies have categorized this lipid biosynthesis inhibitor as harmless to other natural enemies, such as the predators *Episyrphus balteatus* (Degeer) (Diptera: Syrphidae), *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (Schnorbach et al., 2008; Moens et al., 2011), *C. montrouzieri* (Planes et al., 2013) and the parasitoids *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae), *Coccidoxenoides perminutus* Girault (Hymenoptera: Encyrtidae) and *Anagyrus* sp. Near pseudococci (Hymenoptera: Encyrtidae) (Moens et al., 2012). In another study, spirotetramat was classified as moderately toxic and, comparable to our results, it decreased fecundity in *G. occidentalis* (Lefebvre et al., 2011). Also Pratt & Croft (2000) reported that spirotetramat led to 100% adult mortality in *Neoseiulus fallacis* (Acari: Phytoseiidae) adversely affecting all growth stages including fecundity. However, these authors claimed that spirotetramat can be included in an IPM program if applied early in the season because most *N. fallacis* overwinter in the ground cover and later climb into the canopy; only a small proportion overwinters on the trees. Toxic effects on *Typhlodromus pyri* Scheuten (Acari: Phytoseiidae) in the laboratory have been reported by Maus (2008) who also noted that in vineyards spirotetramat appeared to be non-

TABLE 4

Adult survival and sublethal effects of spirotetramat and abamectin at different dilutions of their respective MFRC on reproduction parameters of *M. sexmaculatus* fed on treated prey (*A. pistaciae*) for 30 days, compared to the control (water).

	Spirotetramat [1]			Abamectin [2]	Control
	2/1 MFRC [1]	1/1 MFRC	1/2 MFRC	1/16 MFRC	
Adult survival (%)	100±0	100±0	100±0	80±10	100±0
Egg hatching (%)	85.4±2.6^{b}	91±1.4^{ab}	91.2±1.5^{ab}	90.8±2.3^{ab}	93.9 ±1.2^{a}
Total fecundity	29.0±0.5^{c}	48.1±1.1^{b}	58.9±1.3^{b}	54.2±8.0^{b}	84.9±2.6^{a}
Daily fecundity	2.0±0.1^{d}	3.2±0.2^{c}	3.6 ±0.3^{bc}	4.5±0.2^{b}	5.6±0.3^{a}
Pre-oviposition period (day)	6.2±0.2^{b}	5.8±0.1^{b}	4.0±0.0^{a}	4.2±0.1^{a}	4.0±0.0^{a}

Data are expressed as mean reproduction parameters±SE. Per row, percentages followed by different letters are significantly different (P<0.05, Tukey HSD test).
[1]MFRC for spirotetramat=50 mg/ml
[2]MFRC for abamectin=9 mg/ml

toxic to *T. pyri*, while it is very detrimental to all growth stages of *G. occidentalis* and adversely affects its fecundity (LEFEBVRE et al., 2011). Topical application of spirotetramat did not result in *Bombus impatiens* (Hymenoptera: Apidae) mortality, and it was more effective against insect pests by ingestion than by contact (BRUCK et al., 2009). Although topical application had no effects on bees, high mortality was seen when worker bees chronically ingested field concentrations of spirotetramat (RAMANAIDU & CUTLER, 2013).

Abamectin is an agonist of the GABA (gamma-aminobutyric acid) neurotransmitter in nerve cells and is able to bind to glutamate-gated chloride channels in nerve and muscle cells of invertebrates. BESARD et al. (2010) reported that abamectin was highly toxic and killed 100% of the workers of the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). Also, abamectin was harmful to workers of *B. impatiens* following direct contact, and worker bumblebees consumed less pollen that was supplemented with abamectin (GRADISH et al., 2009). However, KRÄMER & SCHIRMER (2007) considered that abamectin is safe to use with beneficial arthropods under field conditions due to its short environmental persistence, rapid uptake into treated plants and fast degradation of surface residues. Also, HAN et al. (2010) reported that, while beneficials may be killed when treated directly with spray oils or exposed to the vapor phase of essential oils, due to the short term of residual activity, they found no severe effects on the population of phytoseiid mites and other predations. In our laboratory studies, abamectin at 1/1, 1/2, 1/4 and 1/8 of its MFRC was very harmful based on high acute toxicity, so could not be recommended for use in an IPM program. In agreement with our results, other recent studies also categorized abamectin as harmful to predators, such as *Tamarixia triozae* (Hymenoptera: Eulophidae) (LIU et al., 2012), *Ganaspidium nigrimanus* (Kieffer) and *Neochrysocharis formosa* (Westwood) (HERNANDEZ et al., 2011), *Cryptolaemus* sp., *C. sanguinea* and *H. axyridis* (SEAL et al., 2006), *Phytoseiulus persimilis* (Acari: Phytoseiidae) and *Amblyseius fallacis* (Acari: Phytoseiidae) (BOSTANIAN & AKALACH, 2006). We also tested abamectin at lower (sublethal) dosages of 1/16 of its MFRC, and observed severe decreases in total and daily fecundity of *M. sexmaculatus*. KIM et al. (2006) found abamectin caused high adult mortality in *Deraeocoris brevis* (Uhler) (Hemiptera: Miridae) at the full rate, but no mortality at the 10% rate although fecundity was decreased. Similarly, BOSTANIAN &

Akalach (2006) found that abamectin at the rate recommended on the product label reduced the number of eggs laid by individual females of *P. persimilis* and *A. fallacis*. Although in our study, pre-oviposition period was not increased by abamectin exposure, Bin Ibrahim & Sek Yee (2000) reported that abamectin did increase the pre-oviposition period of *Neoseiulus longispinosus* (Acari: Phytoseiidae). Our results suggest that abamectin is not compatible with *M. sexmaculatus* and could not be used in IPM programs based on its strong lethal and sublethal effects. Higher TIER testing under field-related conditions that take account of environmental persistence may be informative.

In conclusion, this study is of importance to the field as our data on acute toxicity effects demonstrated that spirotetramat was harmless and can be compatible with augmentative releases of the coccinellid *M. sexmaculatus*. This confirms the benign profile of spirotetramat when used with predatory coccinellids, but those applying the insecticide need to respect safety periods. Accumulated dosages may reduce the effectiveness of these biological control agents by causing sublethal effects on prey consumption and through reduced fecundity (oviposition). However, we believe that spirotetramat is safe when used at its MFRC, considering that is also metabolized and diluted before reaching the natural enemies; the coccinellids are only maximally exposed to the MFRC at the moment of spraying. In marked contrast, abamectin was harmful and seems not to be compatible with *M. sexmaculatus* due to strong lethal and sublethal effects. However, further testing under more field-realistic conditions may be useful as these would also take environmental persistence into account.

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Trophic interactions between two neustonic organisms: insights from Bayesian stable isotope data analysis tools

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ABSTRACT. The by-the-wind sailor *Velella velella* (Linnaeus, 1758) and its predator, the violet snail *Janthina globosa* (Swainson, 1822) are both floating neustonic organisms. Despite their global oceanic distribution and widespread blooms of *V. velella* in recent years, many gaps remain in our understanding about prey/predator interactions between these two taxa. Using stable isotope ratios of carbon and nitrogen, we aimed to study the trophic relationship between *V. velella* and *J. globosa* and investigate diet variation of *V. velella* and *J. globosa* in relation to individuals' size. Bayesian approaches were used to calculate isotopic niche metrics and the contribution of *V. velella* to the *J. globosa* diet. Our data showed that the isotopic niche of *V. velella* differed markedly from that of *J. globosa*. It was larger and did not overlap that of the *J. globosa*, indicating a more variable diet but at a lower trophic level than *J. globosa*. The isotopic niche of *V. velella* also varied according to the size class of the individual. Small individuals showed a larger isotopic niche than larger animals and low overlap with those of the larger individuals. *J. globosa* displayed very low isotopic variability and very small isotopic niches. In contrast, there were no isotopic composition nor isotopic niche differences between *J. globosa* of any size. This very low isotopic variability suggested that *J. globosa* is a specialist predator, feeding, at least in this aggregation, principally on *V. velella*. Moreover, outputs of a stable isotope mixing model revealed preferential feeding on medium to large (> 500 mm^2) *V. velella* colonies. While our isotopic data showed the trophic relationship between *V. velella* and *J. globosa*, many questions remain about the ecology of these two organisms, demonstrating the need for more fundamental studies about neustonic ecosystems.

KEY WORDS: *Janthina globosa*, *Velella velella*, Mediterranean Sea, neuston, SIBER, SIAR

INTRODUCTION

The by-the-wind sailor *Velella velella* (Linnaeus, 1758) and the violet snail *Janthina globosa* (Swainson, 1822) are both neustonic organisms, i.e., organisms that live upon the upper surface of the ocean and inland waters or beneath its surface film (see definition review by MARSHALL & BURCHARDT, 2005). The colonial *V. velella* (Cnidaria, Hydrozoa, Anthoathecata) floats partly in and partly out of the water whereas *J. globosa* (Mollusca, Gastropoda) is found just beneath the surface, floating with the head pointing down. Both possess floating structures (i.e., chitinous float and bubble raft, respectively), both are unable to swim, and both are passively transported by winds and surface currents. They accumulate in oceanic divergences, where other floating and positively buoyant organisms, such as fish eggs or macrophyte rafts, also concentrate (ZAITSEV, 1971; MARSHALL & BURCHARDT, 2005; PURCELL et al., 2012). These two offshore oceanic species have a worldwide distribution and may sometimes be found stranded in vast numbers on beaches (WILSON & WILSON, 1956; KEMP, 1986). In recent years, widespread blooms of *V. velella* have been observed (PURCELL et al., 2015).

Velella velella is a zooplankton feeder, preying actively on diverse planktonic taxa (e.g., copepods, fish larvae), fish eggs or organisms associated with floating macroalgal rafts (Purcell et al., 2012). Moreover, it hosts symbiotic zooxanthellae, containing chloroplasts (Banaszah et al, 1993). Therefore, it has a relatively varied diet, mixing diverse animal prey and, potentially, symbiotic inputs.

Janthinids are considered to be strict carnivores highly specialised in the consumption of neustonic cnidarians (essentially *V. velella*, the blue button *Porpita porpita* and the Portuguese man-of-war *Physalis physalis*; Bieri, 1966). This has been shown in both laboratory and field conditions, but only through discrete observations of ingestion. Moreover, it is not established whether all individuals of a particular population of a *Janthina* species have exactly the same diet or if variability may occur, for example across individuals of different size. Overall, many gaps remain in our understanding of prey/predator interactions between these two taxa.

Stable isotope ratio measurements are now a classical method used to delineate trophic relationships and to study animal diets (DeNiro & Epstein, 1981). This technique relies upon the fact that the isotopic composition of consumer tissues is the weighted average of the isotopic composition of its food sources, modified by the net isotopic fractionation between diet and animal tissues. Isotopic fractionation (i.e., isotopic composition changes between a substrate and a product, or between two physical states for example) is the result of isotopic effects (i.e., small differential physico-chemical comportments of each isotope), due to mass difference between isotopes. More recently it has been proposed that the variability in isotopic composition of a population or a species (i.e., its isotopic niche) may be used as a proxy to assess the trophic niche of this population or species, and/or the degree of individual specialisation in the population (Bearhop et al., 2004). This isotopic niche concept has also been developed considerably through diverse numerical methods (Matthews & Mazumder, 2004; Layman et al., 2007; Newsome et al., 2007; Jackson et al., 2011).

Using stable isotope ratios and Bayesian numerical tools, the goals of this study were: (1) to study the trophic relationship between *V. velella* and *J. globosa* using trophic biomarkers; (2) to assess the degree of specialism exhibited by *J. globosa* and (3) to investigate potential differences in feeding habits of individuals of *V. velella* and *J. globosa* of different sizes.

MATERIAL AND METHODS

Sample collection and preparation

Velella velella and *Janthina globosa* were sampled on 23 May 2012 in Calvi Bay (Corsica), from large accumulations present in the surface waters of the harbour of the STARESO oceanographic station (University of Liège). To the best of our knowledge, this was the first time that *J. globosa* had been observed in Calvi gulf since being recorded there by the University of Liège in 1968. It does not belong to the neuston normally inhabiting the bay (Collard et al., 2015), so the organisms probably came from offshore areas and passively accumulated in the bay. This exceptional event gave us an opportunity to sample 73 hydrozoan colonies and 74 gastropods, encompassing all size classes observed in the swarm, composed of thousands of individuals. Specimens were sampled using a landing net, manually separated and conserved individually at -28°C until further analysis.

V. velella float length and width were measured to the nearest mm and float area was calculated assuming an elliptical shape using the following formula: $A = \pi ab$, where a is half the length and b half the width of the float. *Janthina globosa* aperture width was measured as a proxy of shell size. *V. velella* were freeze-dried and analysed as a whole after being reduced to homogeneous powder. Gastropod individuals were dissected to separate the foot muscle from other organs. Muscle samples were then freeze-dried and reduced to homogeneous powder for isotopic analysis.

Isotopic measurements

Isotopic ratios of carbon and nitrogen were measured on IR-MS (Isoprime 100, Isoprime, UK) coupled with an N-C-S elemental analyser (Vario Microcube, Elementar, Germany). Stable isotope ratios were expressed in δ notation according to Coplen (2011). Certified materials were IAEA-N2 ($\delta^{15}N$ = +20.30 ± 0.20 ‰) and IAEA C-6 (sucrose) ($\delta^{13}C$ = –10.80 ± 0.47 ‰). Repetitive measurements of glycine ($\delta^{15}N$ = 2.25 ± 0.3 ‰; $\delta^{13}C$ = -47.5 ± 0.3 ‰) were also used to calibrate isotopic data and as an elemental standard. One of the samples was randomly selected and analysed multiple times (once every 15 analyses). Repeatability of these replicate measurements was 0.3 ‰ for both $\delta^{13}C$ and $\delta^{15}N$. Elemental data are expressed in %Dry Mass, and C/N ratios are weight-based.

Statistical analysis

A Mann-Whitney U test was used to test differences between the stable isotope compositions of the two consumers.

Individuals of *V. velella* and *J. globosa* were, *a posteriori*, attributed to different size classes, based on float area (0-500, 501-1000; 1001-1500 mm^2) and aperture width (10-13, 14-18, 19-22 mm), respectively. Allocation to size classes was done by dividing the size range by three, representing small, medium and large individuals in the sampled raft. This was necessary to run the SIAR model. We believe it is of ecological relevance to divide size range into small, medium and large classes and that these size classes reflect the size range observed in the raft. Differences among stable isotopic compositions of respective sizes classes were tested using a non-parametric Kruskal-Wallis test, because conditions for a parametric approach were not present for all groups. Dunn's Multiple Comparison Tests were used to assess pairwise differences when Kruskal-Wallis revealed statistically significant effects. All test results were considered as significant when p was ≤ 0.05. Statistical analyses were conducted using Prism 5.04 (GraphPad Software, La Jolla, USA).

SIBER modelling

Isotopic niche parameters were computed using SIBER (Stable Isotope Bayesian Ellipses in R; Jackson et al., 2011) package (version 2.0) in R 3.2.2 (R Development Core Team, 2008). SIBER was used to generate bivariate standard ellipses that represent core isotopic niches of consumers. Areas of the ellipses associated with each species (SEA_B) were computed using Bayesian modelling (10^6 iterations), and direct pairwise comparisons of SEA_B were performed. Model solutions were presented using credibility intervals of distributions of probability density function.

SIAR Modelling

The stable isotope mixing model SIAR (Stable Isotope Analysis in R; Parnell et al., 2010) was used to estimate the relative contribution of different *V. velella* size classes (isotopic sources) to the diet of *J. globosa*. SIAR 4.2 was fitted in R 3.2.2., including isotopic compositions of each individual, isotopic compositions of food sources (mean ± SD) and trophic enrichment factors (TEFs; expressed as mean ± SD) that correspond to the net isotopic composition change between a consumer and its ingested food source(s).

Here, TEFs for both isotopic ratios were derived from our data using the difference between individual measurements (n=74) of isotopic composition of muscle of *J. globosa* and the average isotopic composition of *V. velella*. Individual TEFs were then averaged to obtain a mean TEF (and associated standard deviation) to be introduced into the SIAR model. Food sources for *J. globosa* were the different size classes of *V. velella* (see above). Model was run with 10^6 iterations and burn-in size was set as 10^5. Model solutions were presented using frequency histograms of probability density functions (Parnell et al., 2010).

RESULTS

$\delta^{15}N$ and $\delta^{13}C$ values of *V. velella* ranged from 1.1 to 4.4 ‰ and from -20.2 to -18.6 ‰, respectively (Fig. 1a). They differed significantly from those of *J. globosa* (Mann-Whitney U, $p<0.001$ for both stable isotope ratios). $\delta^{15}N$ and $\delta^{13}C$ values of *J. globosa* ranged from 4.8 to 6.3 ‰ and from -19.3 to -18.7 ‰, respectively.

TEF values for *J. globosa* were 2.3 ± 0.3 ‰ and 0.2 ± 0.1 ‰ (mean ± S.D., n= 74) for $\delta^{15}N$ and $\delta^{13}C$, respectively.

There was no overlap between the isotopic niches of *V. velella* and that of *J. globosa* (Fig. 1a). Standard Ellipse Area (SEA) of *V. velella* was greater than that of *J. globosa* (0.574 *vs.* 0.106 ‰^{2}). This is confirmed by SEA_B estimation, which showed that, in more than 99.99 % of the solutions generated by the model, ellipses for *V. velella* were greater than those calculated for *J. globosa*.

$\delta^{15}N$ and ^{13}C values for the different *V. velella* size classes differed significantly (Kruskal-Wallis test, $p < 0.001$; Fig. 1b). The smallest *V. velella* (< 500 mm^2) displayed significantly lower $\delta^{15}N$ and more negative $\delta^{13}C$ values than those of the other two size classes (Dunn's Multiple Comparison Tests, $p < 0.01$ for all). $\delta^{15}N$ and $\delta^{13}C$ values of medium and large size

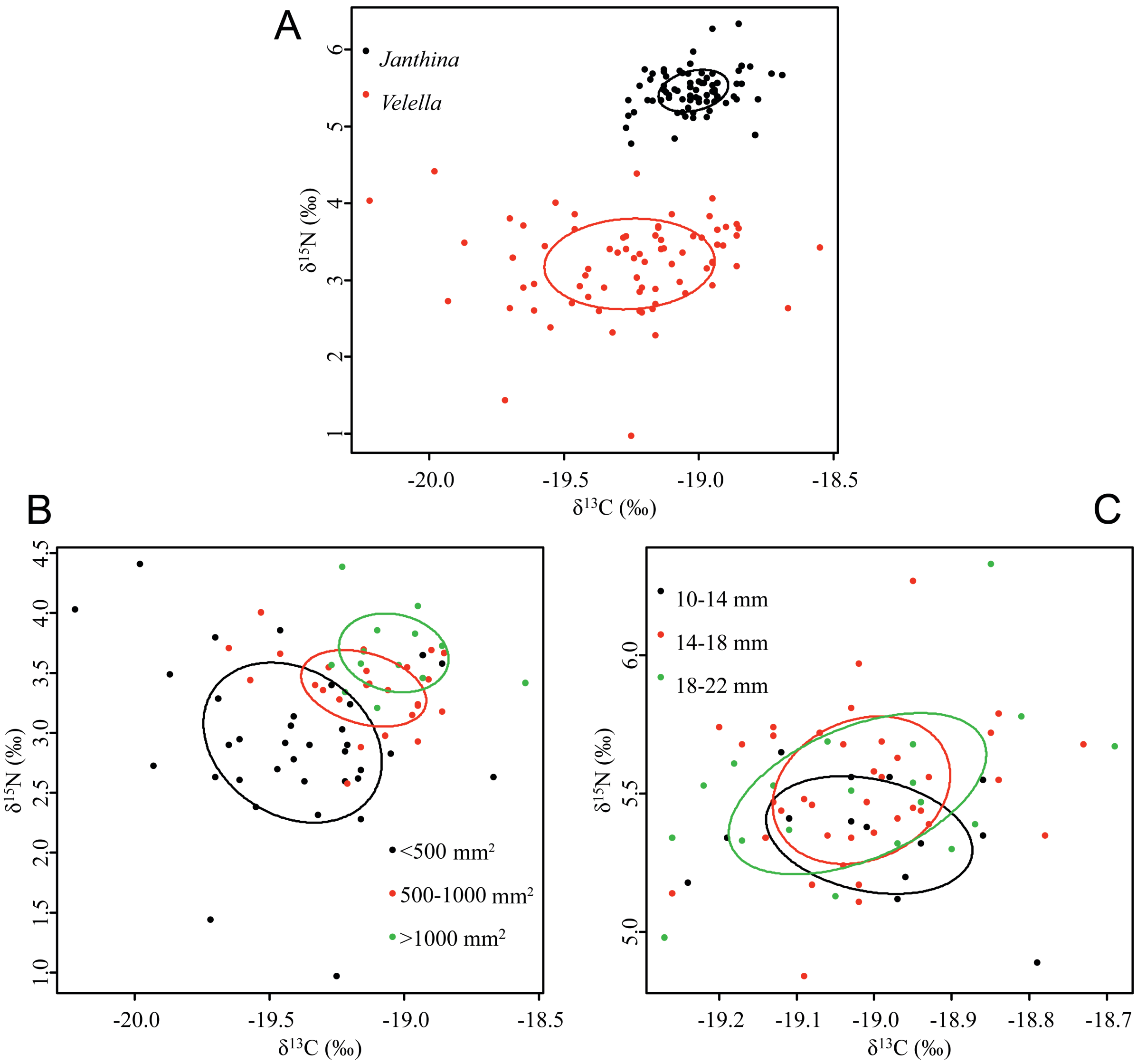

Fig. 1. – Stable isotope compositions of *V. velella* and *J. globosa*. Symbols are individual measurements, and lines are are bivariate standard ellipses that represent the core isotopic niches of consumers. **A**. *V. velella* vs. *J. globosa*. **B**. Different size classes of *V. velella*. **C**. Different size classes of *J. globosa*.

classes did not differ significantly (Dunn's Multiple Comparison Tests, $p \geq 0.5$).

SEA of small *V. velella* was greater than for medium and large individuals (0.654 vs. 0.212 and 0.190 ‰^{2}, respectively; Fig. 1b). SEA_B calculations suggested that this was true in over 99.99 % of model runs (Fig. 2b). SEAs of small and medium *V. velella* overlapped by 0.054 ‰^{2} (i.e., about 8 % of small individual SEAs). SEAs

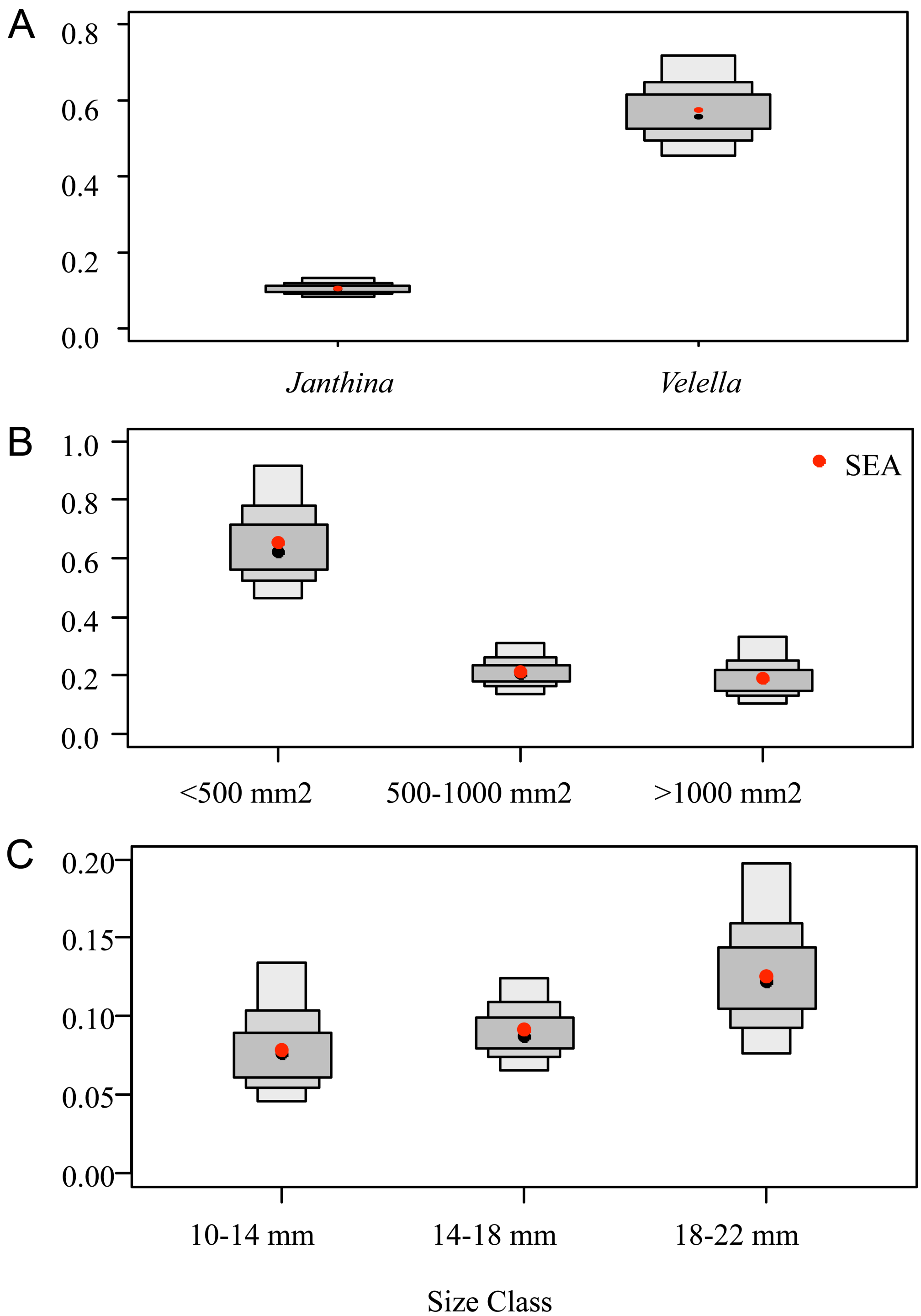

Fig. 2. – Boxplots of model-estimated bivariate standard ellipse area (SEA_B). **A**. *V. velella* vs. *J. globosa*. **B**. different size classes of *V. velella*. **C**. different size classes of *J. globosa*. Dark, median and light grey boxes are respectively the 50 %, 75 % and 95 % credibility intervals of probability density function distributions of the model solutions, and black dots are the modes of these distributions. Red dots are the SEA values associated with each group.

of medium and large individuals overlapped by 0.62 ‰^{2} (i.e., about 30 % of medium SEAs). There was no overlap between SEAs of small and large individuals.

There was no significant difference between the $\delta^{15}N$ and $\delta^{13}C$ values of the three mollusc size classes (Kruskal-Wallis test, $p \geq 0.05$; Fig. 1c). Although SEA of individual *J. globosa* seemed to increase according to size (0.079, 0.091 and 0.125 ‰^{2} for small, medium and large size classes, respectively; Fig 1c), SEA_B did not differ between the three size classes (Fig. 2C). Overlap between SEAs of the three size classes of *J. globosa* was very large (from 0.051 to 0.092 ‰^{2}). Overlap between small and medium janthinids represented 65 % of the SEAs of small janthinids (Fig. 1c). Overlap between medium and large janthinids represented 100 % of the SEA of medium janthinids (Fig 1c). Overlap between small and large janthinids represented 72 % of the SEA of small janthinids.

Because there was no significant difference between the stable isotopic composition of medium and large *V. velella*, SIAR modelling was run with two potential food sources: *Velella* <500 mm^2 and *Velella* >500 mm^2. According to model outputs, the two size classes did not contribute equally to the janthinid diet (Fig. 3). The contribution of medium and large *V. velella* was greater than that of small individuals in

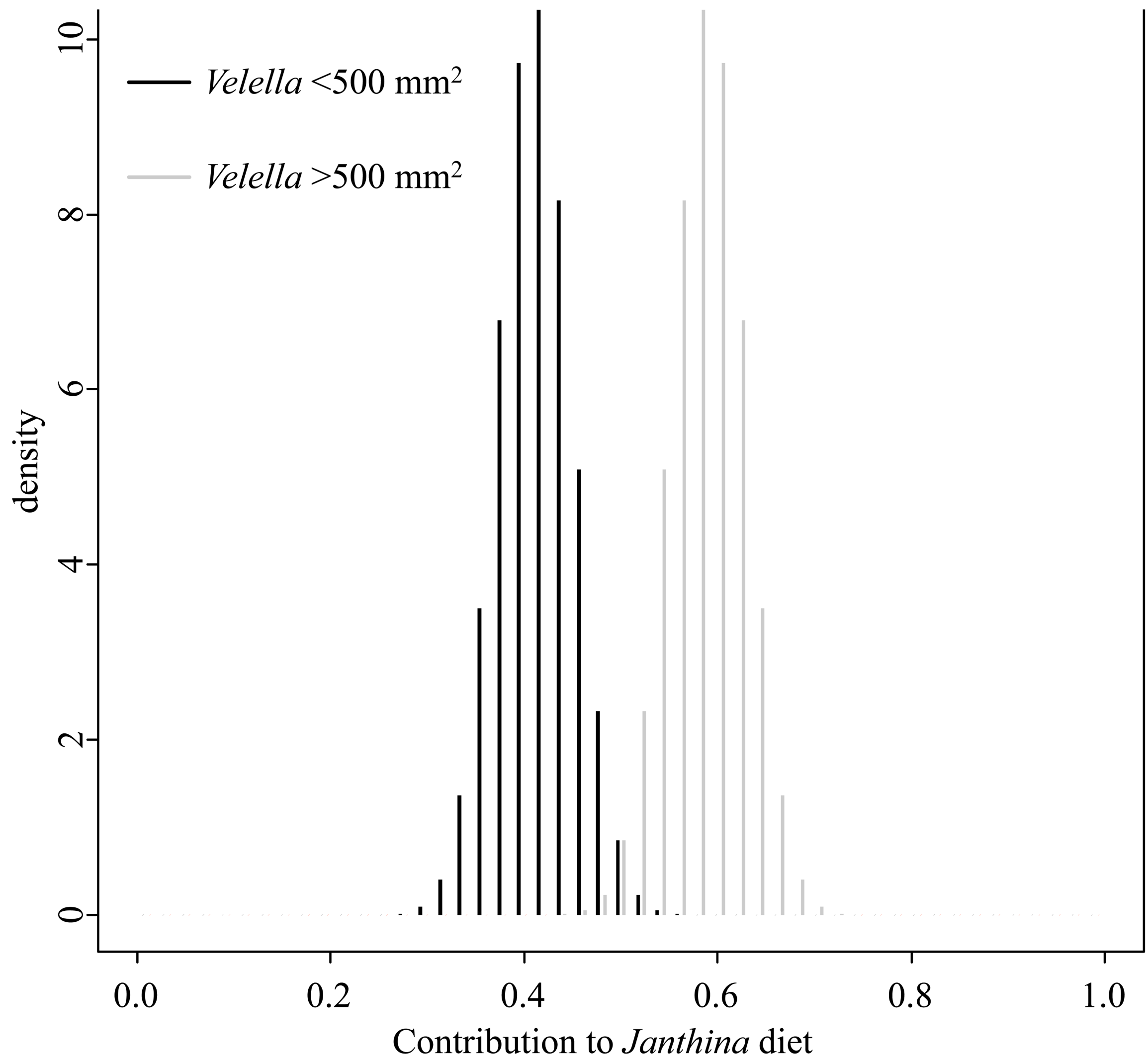

Fig. 3. – Contribution of small and medium/large *V. velella* to the *J. globosa* diet, computed using SIAR. Data are presented as frequency histograms of probability density functions of the model solutions.

98.77 % of model solutions. The contribution of small *V. velella* to the *J. globosa* diet ranged from 0.25 to 0.55 (mode: 0.46), while for medium and large *V. velella,* it ranged from 0.40 to 0.75 (mode: 0.58).

DISCUSSION

Our isotopic data showed the trophic relationship between *V. velella* and *J. globosa*, confirming previous results from stomach content examinations and feeding observations (e.g., BIERI, 1966). The nutritional quality of *V. velella* as a food for *J. globosa* could appear questionable because jellyfish are generally considered to be of low nutritional quality (e.g., BULLARD & HAY, 2002). However, C/N ratios of *V. velella* were relatively close to C/N ratios of *J. globosa* muscles (4.5 ± 0.2 vs. 3.6 ± 0.1, respectively) indicating that *V. velella* could be a suitable food source. Indeed, this C/N value indicates a high protein content and matches a previous observation for the closely related species *Porpita porpita* (BULLARD & HAY, 2002), another neustonic species consumed by janthinids. Protein contents of *P. porpita* were much higher (18 $mg.ml^{-1}$) than in other gelatinous plankton species (< 0.1 $mg.ml^{-1}$ for common schyphozoans such as *Aurelia* sp.) making them valuable prey for predatory organisms despite their nematocyst protection (BULLARD & HAY, 2002). Nutritional value and the presence/absence of nematocysts are the main parameters explaining the consumption (or not) of gelatinous plankton by predators (BULLARD & HAY, 2002). *V. velella* colonies have numerous small gasterozoids with venomous nematocysts to defend the colony and to capture prey. It seems that, in common with other cnidarian-eating specialists such as the leathery turtle *Dermochelis coriacea*, the loggerhead sea turtle *Caretta caretta* or the moonfish *Mola mola* (CARDONA et al., 2012), *J. globosa* is able to tolerate nematocyst attacks. Despite these poisonous cells, janthinids may target the soft tissues of the biggest *V. velella* (BAYER, 1963), largely ignoring sail and float. This could be a way to optimise food quality, as the chitinous float and sail of *V. velella* are potentially less palatable than the colony's soft tissues (BAYER, 1963).

The trophic enrichment factors (i.e., the difference between the stable isotope composition of a consumer and the stable isotopic composition of its food) observed here are in the range reported for carnivorous marine invertebrates (MCCUTCHAN et al., 2003). The TEF values calculated here are based on the difference between isotopic compositions of individual *J. globosa* and the average $\delta^{15}N$ of *V. velella*. This is only truly valid if *J. globosa* feed exclusively on *V. velella* in the study area and if they have only been feeding on *V. velella* for a long period preceding the sampling. As *V. velella* is the most important neustonic hydrozoan in this area, and considering the extent of the sampled raft (i.e., thousands of *V. velella*), we consider that these assumptions were valid in this case but we cannot totally exclude that other food sources are occasionally eaten by *J. globosa*. Another incertitude in this calculation is the fact we averaged a global *V. velella* isotopic composition without taking into account the possibility of size selectivity by *J. globosa* (see modelling results below), and differences in isotopic composition of different *V. velella* size classes. Looking at nitrogen TEFs, these appear comparable to those measured between the schyphomedusa *Chrysaora plocamia* and its parasitic amphipod *Hyperia curticephala* (2.3 ± 0.3 vs. 1.6 ‰, respectively; RIASCOS et al., 2015), while carbon TEFs seem lower (0.2 ± 0.1 vs. 1 ‰) perhaps in relation to good nutritional quality of *V. velella*. For ^{15}N, in comparison to other molluscs, TEFs are comparable with the upper range, yet higher than values measured for herbivorous gastropods (0.4 to 2.0 ‰; CHIKARAISHI et al., 2007). TEFs measured here are also markedly different from TEFs measured for the bivalve *Mytilus edulis* (2.2 ‰ and 3.8 ‰ for carbon and nitrogen, respectively; DUBOIS et al., 2007). This observation supports the hypothesis that diet type (here carnivorous vs. herbivorous or suspension feeder) is often more reliable in explaining TEF variability than phylogeny or life environment (Caut et al., 2009).

The isotopic niche of *V. velella* differed markedly from that of *J. globosa*. It was larger and did not overlap, indicating a more variable diet but at a lower trophic level than *J. globosa,* as indicated by $\delta^{15}N$ values. *V. velella* is an opportunistic zooplankton feeder, eating copepods, fish eggs and larvae and other mesozooplanctonic organisms (Purcell et al., 2012). Nevertheless, when possible, it shows positive selection of some zooplankton items (fish larvae and copepods; Purcell et al., 2012). Moreover, it could derive a part of its organic matter from its symbiosis with zooxanthellae (Banaszah et al., 1993), which could contribute to enlarging its isotopic niche and lowering its trophic level.

Nevertheless, the isotopic niche of *V. velella* varied according to size: small individuals showed a wider isotopic niche than that of larger individuals, and showed low to no overlap with those of larger individuals. In addition, $\delta^{15}N$ values were lower for the smallest *V. velella* than for the two other size classes. There are other examples among jellyfish of isotopic variability according to size, related to diet or trophic level shift (e.g., Fleming et al., 2015; Riascos et al., 2015). Here, smaller *V. velella* most likely have a more diversified diet that larger ones but feed at a lower trophic level (smaller prey for example). Indeed, Fleming et al. (2015) have suggested that jellyfishes of different sizes present simultaneously in a water column occupy different trophic positions in the food web.

A second, not exclusive, explanation is that the isotopic composition of smaller individuals may not be at isotopic equilibrium with their current food. *V. velella* individuals we sampled were from a neustonic colony, composed of many individuals and reproducing asexually. However, colony founders are produced sexually from medusae living in deep waters, between 100 and 800 m depth in the Mediterranean (Larson, 1980). Founders begin their life at that depth and, therefore, their initial isotopic composition reflects their feeding in the epipelagic zone where isotopic composition of prey may differ from the euphotic zone. Because of tissue renewal, it takes time to go from an initial isotopic composition to one reflecting that of a changed diet (Matthews & Mazumder, 2005). This additional source of variability linked to habitat may also explain the larger isotopic niche of small individuals (Flaherty & Ben-David, 2010). Moreover, each individual may originate from different epipelagic areas and join the neustonic raft at different moments and in different locations. Neustonic organisms indeed passively accumulate according to wind pattern and Langmuir cell organisation (Zaitsev, 1971). Finally, *V. velella* could partly rely on a symbiosis with zooxanthellae to find its nutritional balance (Banaszah et al., 1993). Although these symbionts are already present in medusae and larvae (Banaszah et al., 1993), the contribution of these symbiotic relationships to the diet of *V. velella* may vary according to size.

In contrast to *V. velella*, the *J. globosa* displayed a very low isotopic variability (± 0.3 and 0.1 ‰ S.D. for $\delta^{15}N$ and $\delta^{13}C$, respectively, n= 74). This variability is lower than the instrument repeatability of our measurement system (cf. “Material and Methods” section). It is largely inferior to isotopic variability measured for other planktonic or benthic organisms sampled from Calvi bay (Lepoint et al., 2000; Michel et al., 2014), or for other predatory marine gastropods such as Terebrids (Fedosov et al., 2014). Moreover, it was impossible to see any variability in relation to the size of our individuals. Commonly, in both fish and invertebrates, diet changes according to age (change of prey and/or trophic level; Jennings et al., 2008; Frederich et al., 2010; Riascos et al., 2015). As indicated by the absence of ^{15}N variability, in this sampled raft, *J. globosa* did not shift to other prey or other trophic levels during their growth. Laboratory observations report the existence of cannibalism by larger individuals on smaller ones (Bayer, 1963). It was apparently not common in our population, since the trophic level (i.e. $\delta^{15}N$)

did not increase according to individual size and $\delta^{15}N$ values were consistent with one trophic level increase between *V. velella* and *J. globosa*.

Most isotopic niche studies focus on generalist species or populations, trying to determine the degree of individual specialisation or isotopic niche area and overlap (Layman & Allgeier, 2012; Fleming et al., 2015). Bearhop et al. (2004) hypothesized that a specialist population (or species) composed of individuals feeding on the same unique food source, or a generalist population composed of generalist individuals all feeding on exactly the same food sources, should display almost no isotopic variability, compared to a generalist population composed of individuals feeding on different food sources. This has been demonstrated experimentally by diet-controlled experiments (Fink et al., 2012). The very low isotopic variability recorded here for *J. globosa* supports the idea that the hypothesis of Bearhop et al. (2004) applies also for this natural population. Nevertheless, the extremely low isotopic variability observed here (i.e. close to 0) cannot be attributed to a generalist population composed of generalist individuals (i.e. with a diet composed of different food sources) because this would imply that every *J. globosa* individual fed on exactly the same food sources in the same proportion. In natural populations, when more than one food source is eaten, there is always an isotopic variability link to small diet difference between individuals. This could indicate that our population was composed of individuals feeding almost exclusively on the same unique food source, namely the *V. velella*.

Here, in this population, the diet of *J. globosa* could be invariable regardless of age and could be exclusively one prey. However, we may hypothesise that inter-population variability in feeding habits may occur. Gut content examinations have shown that other pelagic prey may be consumed (*P. porpyta*, *Physalis physalis*, tropical pelagic anemones; Bieri, 1966). In addition, cannibalism has also been observed (Bayer, 1963), although not demonstrated by our data set (i.e., no increase of $\delta^{15}N$ with individual size). We sampled just one *J. globosa* population associated only with abundant numbers of *V. velella* meaning individuals were probably easily able to find sufficient food by preying exclusively on *V. velella.* Nevertheless, these two species are cosmopolitan and may encounter different life conditions (e.g., a different mix of neustonic species, less availability of prey and starvation), depending of the area where they live (tropical vs. temperate for example) and raft history.

SIAR modelling showed an effect of *V. velella* class size on diet of *J. globosa* related to size of *V. velella*. TEFs used to run the model did not taken into account the different size classes of *V. velella*. Nevertheless, we believe that any possible uncertainty arising as a consequence of TEF variability is taken into account by the standard variation introduced around our average TEF, and overall believe that our mixing model remains valid against this potential incertitude. SIAR modelling demonstrated that *J. janthina* feed preferentially on bigger *V. velella*. *Janthina* spp. may chew an entire individual of *V. velella* within a few hours (Bayer, 1963). But it has also been observed that *Janthina* spp.only graze on the soft part of *V. velella* leaving the chitinous part of the colony (Bayer, 1963). Large *V. velella* individuals offer more soft tissues than little ones, which may explain this preference. Moreover, *J. globosa* were also observed discarding their bubble raft, climbing on colonies of *V. velella* to graze on their soft parts and then reforming a new bubble raft (Bayer, 1963). Such behaviour is probably only possible when *V. velella* are large enough to support the weight of the janthinids. Indeed, janthinids are unable to swim and sink to their death when separated from their bubble raft (Bayer, 1963). Feeding behaviour could therefore explain the preferential consumption of medium to large *V. velella* colonies. The neustonic ecosystem is omnipresent, as more than 70 % of Earth's area is covered with water. Here, we showed that much is yet to be discovered, even about the basic ecology of cosmopolitan, widespread species. Neuston currently receives little attention, and most of the functioning and biodiversity of these systems remains unknown. This demonstrates a need for more fundamental studies on neustonic ecosystems and their ecology.

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SHORT NOTE

Preferential use of one paw during feeding in the subterranean rodent *Ctenomys talarum*

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Initially believed to be a uniquely human characteristic, the preference to use one extremity for carrying out diverse activities, such as feeding or self-grooming, has been observed in several groups of vertebrates and even in some invertebrates (1,2,3). These behavioral asymmetries, which may reflect differences in the roles of the two brain hemispheres, are classified according to their occurrence in the individuals at the population level: no asymmetry, when all individuals prefer to use both the left and the right limb with equal probability; individual-level asymmetry, when some individuals of the population prefer to use one extremity while others prefer to use the other limb (no asymmetry at the population level); and population asymmetry, when most of the individuals prefer to use either the left or the right limb (3).

Among mammals, rodents constitute one of the most studied groups in the field of limb preferences. However, there are still controversies about how to classify this mammalian order basing on their paw lateralization. While some studies suggest that paw preferences in rodents show individual, but not population-level asymmetry (4,5,6), others indicate a population-level right handedness (7,8), although individual characteristics, such as sex, reproductive condition or strain, and even environmental factors or the kind of testing protocol used, appear to influence the degree and direction of lateralization in these species (3). At this point, it should be noted that most of the studies were carried out on “model organisms”, such as *Rattus norvergicus* and *Mus musculus*, while investigations of paw preferences in wild species of rodents are comparatively scarce.

Ctenomys talarum (Thomas, 1898), commonly named as tuco-tuco, is a solitary species of subterranean rodent that inhabits sand dune belts in Buenos Aires Province, Argentina (9). This herbivorous rodent forages aboveground, when tuco-tucos emerge from burrow openings and travel short distances (less than one meter) to cut grasses and perennial forbs growing in the soil. However, the consumption of the collected food occurs inside their tunnels (10,11,12). Despite the difficulty of recording their feeding behavior in their natural habitat, laboratory observations suggested that individuals of this species prefer to use one paw when manipulating and consuming the leaves and stems. The feeding behavior of this subterranean rodent comprises several different steps that include catching food items with the mouth and one or both hands, cutting them into small pieces with the teeth, the removal of the superficial layers of the stems with the teeth while rotating the stems with the hands, and taking the leaves or stems to the mouth to ingest them after mastication.

The main aim of this study was to explore whether this species of wild subterranean rodent displays forepaw preferences while feeding, and if so, whether this lateralization occurs at the individual and/or population level. The results of this study will add valuable information to our understanding of laterality in mammals in general and in rodents in particular, a group where a profound bias exists in terms of the number and diversity of species that have been studied.

Adult *C. talarum* individuals (n=14) were captured at Mar de Cobo (Buenos Aires Province, Argentina) using live traps set at fresh surface mounds. Then, individuals were carried to the biotherium and housed individually in plastic cages (42 × 34 × 26 cm) with wood shavings as bedding. A fresh supply of vegetables (carrots, sweet potatoes, lettuce and mixed grasses) was provided daily. The animal room was maintained at a thermoneutral temperature (23 ± 1 °C) and natural photoperiod. Relative ambient humidity ranged from 50 to 70%. Before recording the feeding behavior, animals were food-deprived for 24 hr to increase their motivation to eat. As a result, individuals devoted most of the recording time to eating or manipulating food items.

To record tuco-tucos´ feeding behavior, a Plexigas transparent chamber (45 × 30 × 30 cm) was used. Before starting the recordings, the individuals were left inside the testing chamber for 10 min to acclimate to it. Then, several items of *Panicum racemosum* (the most abundant plant species both in the habitat and diet of *C. talarum*) (11) were placed inside the chamber equidistant to the individual's sides, and the feeding behavior was recorded for a single 30 min period with a video camera. Later, videotapes were viewed and the following feeding parameters registered:

a) paw used by tuco-tucos to reach food to cut it.
b) paw used to rotate the plant stems while removing the superficial layers of them with the teeth.
c) paw used to take food to the mouth to eat.

Only clear views of tuco-tucos´ behavior while feeding were used to calculate paw preference. When an animal took a food item with one paw and carried it to its mouth repeatedly without dropping it, this was calculated as a single bout. If the individual passed the same food item from one hand to the other recurrently while eating, the most frequent paw used to carry the food to the mouth was considered to classify the bout and for the analysis. The measure of paw preference was calculated as the number of times the animals used their left, right or both paws to manipulate food items in all recorded bouts. Based on the frequencies of use, paw preference was conferred to the individuals using one paw for at least 66% of times (13). Therefore, tuco-tucos displaying 66% or more left paw uses were classified as left-preferent, those with 33% or less left paw uses were classed as right-preferent, and those with scores between 34% and 65% were classed as ambidextrous (13). Also, handedness index values (HI) and z scores were calculated. The HI value is calculated by dividing the difference between the total number of left and right paw reaches by the sum of them (RP - LP)/(LP + RP). Positive values reflect right hand preferences and negative values indicate left hand preferences. Although there is some controversy about its utility in laterality studies (14), the z score is still one of the most used statistical tests for analyzing handedness. The forepaw preference in each type of feeding behavior for each animal was determined by calculating an individual z score on the basis of the total number of left and right forepaw responses using the binomial test. Z score values of ± 1.96 are the critical values. Based on z scores, individuals are categorized as right-handed (z > 1.96), left-handed (z < -1.96), or ambidextrous (1.96 > z > -1.96) (14).

RESULTS

Paw used by tuco-tucos to catch food to cut it

No individual displayed left-paw preference and only one displayed right-paw preference (Fig. 1). The majority of the individuals (n=9) more frequently used both paws to catch food items to cut them with their teeth, while the others used the right or left paw more often but always less than 66% of the times, clearly suggesting an absence of preference in paw use in this feeding behavior. Values of HI and z scores are represented in Table 1. Since these methods are based only on right and left preferences, they are not very valuable for analyzing this feeding behavior when most of the individuals are using both paws. Even so, it can be seen that most of the tuco-tucos did not display right or left preferences after excluding both paw frequencies.

Paw used to rotate the plant stems while removing the superficial layers of them with the teeth

In all the events collected, the individuals used both paws to remove the superficial layers of the stems.

Paw used to take food to the mouth to eat

Ten out of the 14 studied individuals displayed a clear left-paw preference to take food items to the mouth to eat, although no single animal used the left paw 100% of the time (Fig. 2). The other 4 tuco-tucos did not use any paw more than 66% of the times, being therefore classified as ambidextrous. However, of these four individuals, three more frequently used the left paw, while the last one used both paws likewise. A similar trend was observed in the HI, with 10 individuals showing a strong left-paw preference (mean: -0.65, n = 10). On the basis of individual z scores, eleven tuco-tucos were classified as "left-handed" and the other three as ambiguously "handed". Based on this classification, statistical analysis indicated that the three categories (left-, right-paw preferent or ambidextrous) were not similarly represented (chi-square test, df = 2, $p < 0.01$). Also, when analyzing if proportions of left-pawed and non left-pawed individuals were equally represented, the analysis revealed statistical differences, indicating a left bias for this task (chi-square test, df = 1, p= 0.03).

The historical view that no other animal species display preferences in the use of one limb in a similar way to that observed in humans has been refuted in light of new evidence that has demonstrated the preferential use of one extremity in several species of vertebrates and even invertebrates (2). For example, population-level asymmetries were described in the dog (*Canis familiaris*) although sex differences in the expression of this preference were observed. While male dogs preferred their left paw to remove an adhesive strip from the snout,

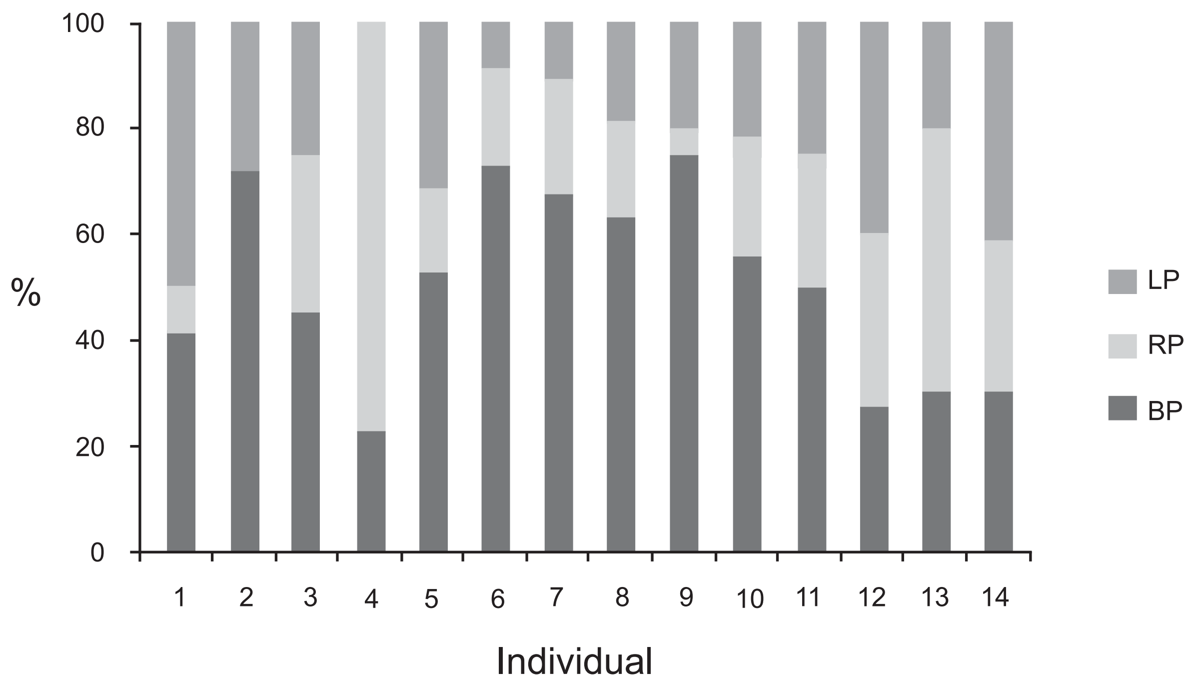

Fig.1. – Percentage of left paw (LP), right paw (RP) and both paws (BP) use by tuco-tucos to catch food to cut it.

TABLE 1
Table setting out the handedness index (HI) and z score for each individual for two of the three feeding behaviors recorded.

	Catch food items		**Eat food items**	
Individual	HI	Z score	HI	Z score
1	0,71	-2,67	-0,42	-2,59
2	1	-2	-0,48	-2,5
3	-0,09	0,3	-0,11	-0,57
4	-1	3,16	-0,55	-2,88
5	0,33	-1	-0,52	-2,18
6	-0,33	0,57	-0,21	-1,04
7	-0,33	0,57	0	0
8	0	0	-0,76	-3,8
9	0,6	-1,34	-0,8	-3,7
10	0	0	-0,5	-2,23
11	0	0	-0,88	-3,63
12	0,09	-0,42	-0,52	-2,6
13	-0,42	1,6	-0,89	-3,9
14	0,16	-1,23	-0,73	-2,84

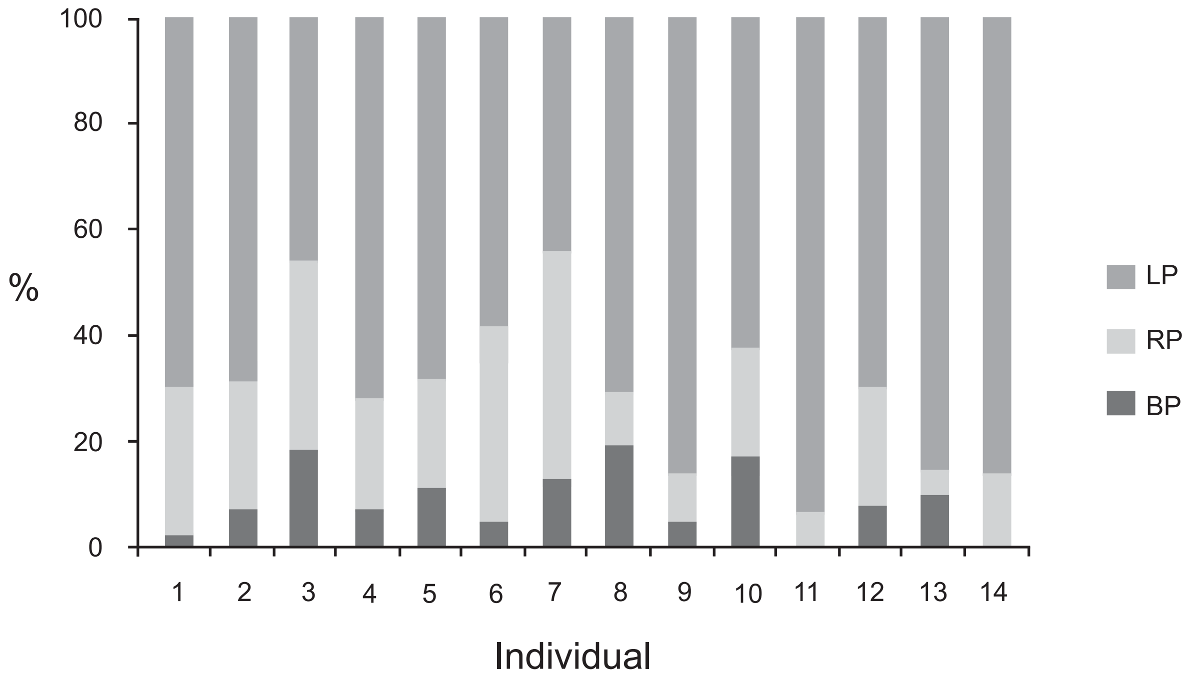

Fig.2. – Percentage of left paw (LP), right paw (RP) and both paws (BP) use by tuco-tucos to take food to the mouth to eat.

females preferred to use the right paw (15). Paw preferences were also observed in the domestic cat (*Felis silvestris catus*) although no population-level asymmetry was recorded, but instead an individual-level asymmetry was observed (13). Using a food handling test, authors observed that 46% of the cats were right-preferent, 44% were left-preferent and 10% were ambilateral, with no differences between male and female cats in the proportions of left and right paw-preferent individuals.

While several studies have addressed the question of paw preference in rodents (see 3), none has previously examined paw preference in a wild species of subterranean rodent. The majority of tuco-tucos analyzed in this study showed a significant left-paw preference for carrying the food items to the mouth, a situation that contrasts with most of the studies in rodents, which provide evidence for a lateralization in paw preference, but in the opposite direction (3,16,17,18). However, and as explained before, testing protocol used and kind of task studied could result in the appearance of different or contrasting results. Therefore, a comparison of different studies of paw preferences in diverse rodent species should be undertaken cautiously.

Regarding the other feeding parameters analyzed, none revealed any preference in paw usage. When rotating plant stems to remove the superficial layers with the teeth, tuco-tucos always utilized both paws, a situation that may reflect the complexity of the task, which requires the use of both paws simultaneously, rather than the absence of paw preferences.

As reviewed by Ströckens et al. (3), the majority of studies suggest that paw preferences in rodents show individual-level asymmetry, but not population level asymmetry. In the case of *C. talarum*, the results of this work provide support for a leftward population-level asymmetry. Nevertheless, as only 14 individuals from one population of *C. talarum* were studied, additional animals should be studied before firm conclusions are drawn regarding this species' paw preference.

In conclusion, this study presents the first evidence for a lateralization in paw use during feeding in a wild species of subterranean rodent. Further research is necessary in order to investigate if this lateralization occurs in various manual tasks and if it is manifest in other populations of this and other species of tuco-tucos.

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