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Heavy metals in livers of raptors from Eastern Poland – the importance of diet composition

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ABSTRACT. Concentrations of Pb, Cd, Ni, Cr and Hg were determined in livers of six species of raptors collected in the area of Eastern Poland. Redundancy analysis (RDA) showed that elevated Hg and Cr concentrations were directly related to feeding on passerines. Raptors that specialised in seizing small mammals as a source of food revealed higher hepatic concentrations of Pb and Cd in comparison with other raptors. Unlike Cd, we found statistically significant differences in the Pb concentrations in livers of Common buzzards as compared to Sparrowhawks. In spite of the fact that both Goshawks and Sparrowhawks hunt birds, only the latter species had accumulated significantly more mercury. The high concentrations of Hg in Sparrowhawks could be related to the use of mercury in antifungal substances for seed dressing.

KEY WORDS: raptors, liver, heavy metals, lead bullets.

INTRODUCTION

In spite of extensive surveys, many aspects of heavy metal accumulation and transmission in ecosystems remain unclear (Helander et al., 2009; Stankovic et al., 2013; Kitowski et al., 2014). Exposure to high levels of heavy metals through diet or other activities can have toxic effects on avian species influencing their hormone and respiratory systems, reproduction and migration (Scheuhammer 1987; Haschek et al., 2013; Williams et al., 2014). Raptors seem to be especially well-suited models for investigating these processes in birds as top predators in food chains, because they also have a wide geographical distribution and are often sedentary (Martin et al., 2008; Castro et al., 2011; Rajamani & Subramanian, 2015; Golden et al., 2016). It has been pointed out that metal concentrations in birds are good indicators of the level of heavy metal concentration in their diet, which is strongly associated with the degree of contamination in the exploited ecosystem (Pain et al., 1997; Castro et al., 2011). Eastern European countries are unfortunately prominent in heavy metal (Pb, Cd, Ni, Cr, Hg) usage (Pacyna et al., 2009; Pacyna et al., 2011) causing a serious strain on all levels of ecosystems, including raptors (Stankovic et al., 2013; Kalisinska et al., 2014). The wide use of lead bullets by hunters in many countries world-wide (Fisher et al., 2006) is another important source of contamination of heavy metals in birds and their predators. The aim of the present study was to determine Pb, Cd, Ni, Cr and Hg concentrations in livers of six raptor species nesting in East Poland. We hypothesized that these raptor species show different levels of heavy metal burdens due to differences in habitat selection and prey.

MATERIALS AND METHODS

The investigated material consisted of the livers (collected from 58 specimens) of the following raptor species: Common buzzard *Buteo buteo* (31 specimens), Sparrowhawk *Accipiter nisus* (10), Goshawk *Accipiter gentilis* (9), Marsh harrier *Circus aeruginosus* (3), Common kestrel *Falco tinnunculus* (3) and White-tailed eagle *Haliaeetus albicilla* (2). The studied birds originated from different habitats in Eastern Poland (Rzeszów, Białystok, Lublin and Warsaw regions). The livers used in this study were obtained from wounded raptors delivered to rehabilitation centres or veterinary clinics close to raptor nesting sites between 2009 and 2012. The raptors either died despite persistent veterinary treatment, or they were untreatable upon delivery and were administered lethal injection. The raptors' total stay in the clinics or rehabilitation centres did not exceed seven days. Following extraction, the livers were stored in freezers at -30°C. Prior to measurement, the livers were freeze-dried and ground in a ceramic mortar. All glassware and utensils were rinsed with tap water, soaked in an acid bath (5M HNO_3) for 24 h, rinsed with demineralised water and dried under a laminar flow hood before use, to minimise the risk of any metal contamination. Weighed portions of the samples (500 ± 1 mg) were dissolved with 10 mL of concentrated HNO_3 (Sigma Aldrich) and subjected to wet-ashing. Mineralisation was carried out using the Microwave Digestion System with optical temperature and pressure monitoring of each individual sample during acid digestion (Berghof Speedwave) in Teflon vials (DAP 100 type). The mineralization process was conducted according to the following scheme: 15 min of temperature rise from room temperature up to 140°C, 5 min at 140°C, 5 min heating from 140°C up to 170°C, 15 min at 170°C, followed by cooling down to room temperature (variable time). The pressure over the whole mineralization process did not exceed 12 bar. After completion of the mineralization process a clear solution was obtained, which was made up to 50 mL with demineralised water (ELGA Pure Lab Classic). In this study, ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) from Thermo Scientific iCAP Series 6500, equipped with a charge injection device (CID) detector was used for element determination. The spectrometer was controlled by the PC-based iTEVA software. The following instrumental parameters were set: RF power generator 1150 W, RF frequency generator of 27.12 MHz, coolant gas flow rate - 16 $L \cdot min^{-1}$, carrier gas flow rate - 0.65 $L \cdot min^{-1}$, auxiliary gas flow rate - 0.4 $L \cdot min^{-1}$, maximal integration time - 15 s, pump rate - 50 rpm, viewing configuration - Axial, replicate - 3, Flush time - 20 s. Analityk - 47: ^{27}Al, ^{75}As, ^{111}Cd, ^{52}Cr, ^{208}Pb, ^{55}Mn, ^{201}Hg, ^{60}Ni, ^{45}Sc, ^{79}Se, ^{88}Sr, ^{51}V, ^{66}Zn in 10% HNO_3 - 100 $mg \cdot kg^{-1}$, multi-element stock solution (Inorganic Ventures) was used as standard.

Redundancy analysis (RDA) was used to establish the relation between heavy metal concentrations and the food habits of the different raptor species. The data required for RDA analysis to rank the habitat and trophic preferences of studied raptors were taken from the literature (Cramp & Simmons, 1980; Newton, 1986; Witkowski, 1989; Village, 1990; Zaccaroni et al., 2008). For statistical analyses, median values were compared using Mann-Whitney and Kruskall-Wallis H tests (Sokal & Rohlf, 1981). Statistical calculations were performed with Statistica, while Canoco 4.5 software was used to visualize some statistical results (ter Braak & Šmilauer, 2002).

Metal concentrations are expressed in mg/kg dry weight (dw). If literature data were expressed as wet mass of a sample, a conversion factor of 4.0 from a wet weight (ww) to a dry mass of liver was used, according to Kalisinska et al. (2004). Liver lead concentrations ≥ 6 mg/kg dw were considered to be diagnostic of elevated exposure resulting in subclinical toxicity. Liver lead concentrations ≥ 15 mg/kg dw were considered as diagnostic of lead poisoning (Franson, 1996). Acute lead poisoning has been observed when liver concentrations exceed 30 mg/kg dw. The background levels of lead are usually far

below 6 mg/kg dw (MARTIN et al., 2008). In the case of cadmium, other authors (BURGAT, 1990; BATTAGLIA et al., 2005) suggested that Cd levels ≥ 3 mg/kg dw in liver might indicate increased environmental exposure.

RESULTS

Trends in concentrations

RDA analyses were performed taking feeding ecology into account, more specifically the type of preferred food and the foraging area. Among the analysed variables, only a food preference for small birds (Food SB) had a significant relationship with lead and mercury concentrations (Lambda-A: 0.09, F = 5.42, p = 0.01; Fig.1). The RDA analyses also showed that elevated concentrations of chromium and mercury were related to feeding on small birds (passerines) as is obvious in the case of Sparrowhawks (Fig. 1; Table 2). Prey captured from water was not a major source of mercury for any of the studied raptors (Fig.1). Feeding on larger avian prey seemed to prevent mercury accumulations, as in Goshawks in contrast to other raptors feeding on small birds. The RDA also showed that investigated individuals with larger body masses tended to accumulate less mercury (for example Common buzzard and White-tailed eagle) (Fig. 1). As is obvious from Fig. 1, the elevated concentrations of cadmium and lead corresponded mostly to the consumption of small mammals as well as to an increasing consumption of medium sized mammals, carrion and game. Buzzards were the only raptor species showing any effect for lead.

Concentrations levels in livers of raptors

The measured concentrations of the various heavy metals in livers of the investigated raptors are presented in Table 1.

Regarding cadmium, mean concentrations of this metal ranged from 0.282 to 0.786 mg/kg dw (Table 1), with a maximum of 3.425 mg/kg dw in one Buzzard. There were no significant differences in hepatic concentrations of cadmium between Goshawks, Sparrowhawks and Buzzards (Kruskall-Wallis H-test: H = 0.719, df = 2, N = 50, p = 0.699). The highest mean level of chromium was also found in Sparrowhawks (Table 1). Significant differences in the hepatic level of Cr were observed between Goshawks and Sparrowhawks (Mann-Whitney U test: Z = 3.67, n_1 = 10, n_2 = 9, p = 0.00024) (Table 1).

The highest individual level of mercury (11.99 mg/kg) was found in a Sparrowhawk. The mean hepatic concentrations of Hg in Goshawks and buzzards were generally much smaller than in Sparrowhawks (Table 1; and statistically significant differences in the concentrations of mercury were found between the two hawk species of the genus *Accipiter sp*. studied here (Mann-Whitney U test: Z = 3.18, n_1 = 10, n_2 = 9, p = 0.004).

Mean levels of nickel varied between 0.234 and 0.343 mg/kg dw, for White-tailed eagles and kestrel, respectively (Table 1). An equally narrow range of Ni concentrations was also observed in Goshawks, Sparrowhawks and Buzzards and, similarly to cadmium, no significant differences were observed between these species (Kruskall-Wallis H-test: H = 0.777, df = 2, N = 50, p = 0.686). Mean hepatic concentrations of lead were significantly lower in Sparrowhawks than in buzzards (Table 1; Mann-Whitney U test: Z= 3.95, n_1 = 10, n_2 = 31, p = 0.00008). In Buzzards, we found two cases with Pb concentrations of 8.61 and 8.58 mg/kg dw, respectively. Such concentrations are considered to be diagnostic of elevated lead exposure, resulting in subclinical toxicity. In a single liver from this species, the hepatic lead level reached 15.31 mg/kg dw indicating lead poisoning.

DISCUSSION

Our study shows that prey preference has an effect on the heavy metal load in raptor species. Below, we compare our results to those of other regions for each heavy metal.

TABLE 1

Descriptive statistics of Cd, Cr, Hg, Ni and Pb concentrations (mg/kg dry weight) in the liver of six raptor species from Eastern Poland including: White-tailed eagle *Haliaeetus albicilla,* Marsh Harrier *Circus aeruginosus,* Goshawk *Accipiter gentilis,* Sparrowhawk *Accipiter nisus,* Common buzzard *Buteo buteo* and Common kestrel *Falco tinnunculus*. The geometric mean and concentration range are given. * = in case of just two measurements, the arithmetic mean is given; SD = standard deviation.

Species	Cadmium (Cd)		Chromium (Cr)		Mercury (Hg)		Nickel (Ni)		Lead (Pb)	
	Geometric mean	SD	Geometric mean	SD	Geometric mean	SD	Geometric mean	SD	Geometric mean	SD
	range:		range:		range:		range:		range:	
White tailed eagles N = 2	*0.290	-	*0.814	-	*0.959	-	*0.234	-	*0.585	-
	0.110 - 0.469		0.465 - 1.163		0.183 - 1.734		0.202 - 0.266		0.544 - 0.625	
Marsh Harrier N = 3	0.282	0.282	0.752	0.259	2.398	2.161	0.300	0.040	0.684	0.139
	0.118 - 0.608		0.576 - 1.050		0.096 - 4.382		0.290 - 0.343		0.530 - 0.810	
Goshawk N = 9	0.519	0.853	0.549	0.064	0.206	0.292	0.295	0.027	1.152	0.810
	0.150 - 1.547		0.448 - 0.654		0.144 - 0.978		0.248 - 0.331		0.650 - 2.851	
Sparrowhawk N = 10	0.552	0. 244	1.220	0.149	2.001	3.428	0.304	0.043	0.350	0.376
	0.101 - 0.991		1.025 - 1.462		0.191 - 11.990		0.262 - 0.383		0.246 - 1.468	
Common Buzzard N = 31	0.786	0.702	0.466	0.183	0.633	1.90	0.247	0.084	1.217	3. 189
	0.145 - 3.425		0.321 - 1.384		0.059 - 9.256		0.092 - 0.390		0.430 - 15.31	
Kestrel N = 3	0.322	0.117	0.640	0.031	0.639	0.723	0.343	0.018	0.728	0.056
	0.156 - 0.410		0.597 - 0.662		0.069 - 1.659		0.321 - 0.364		0.652 - 0.787	

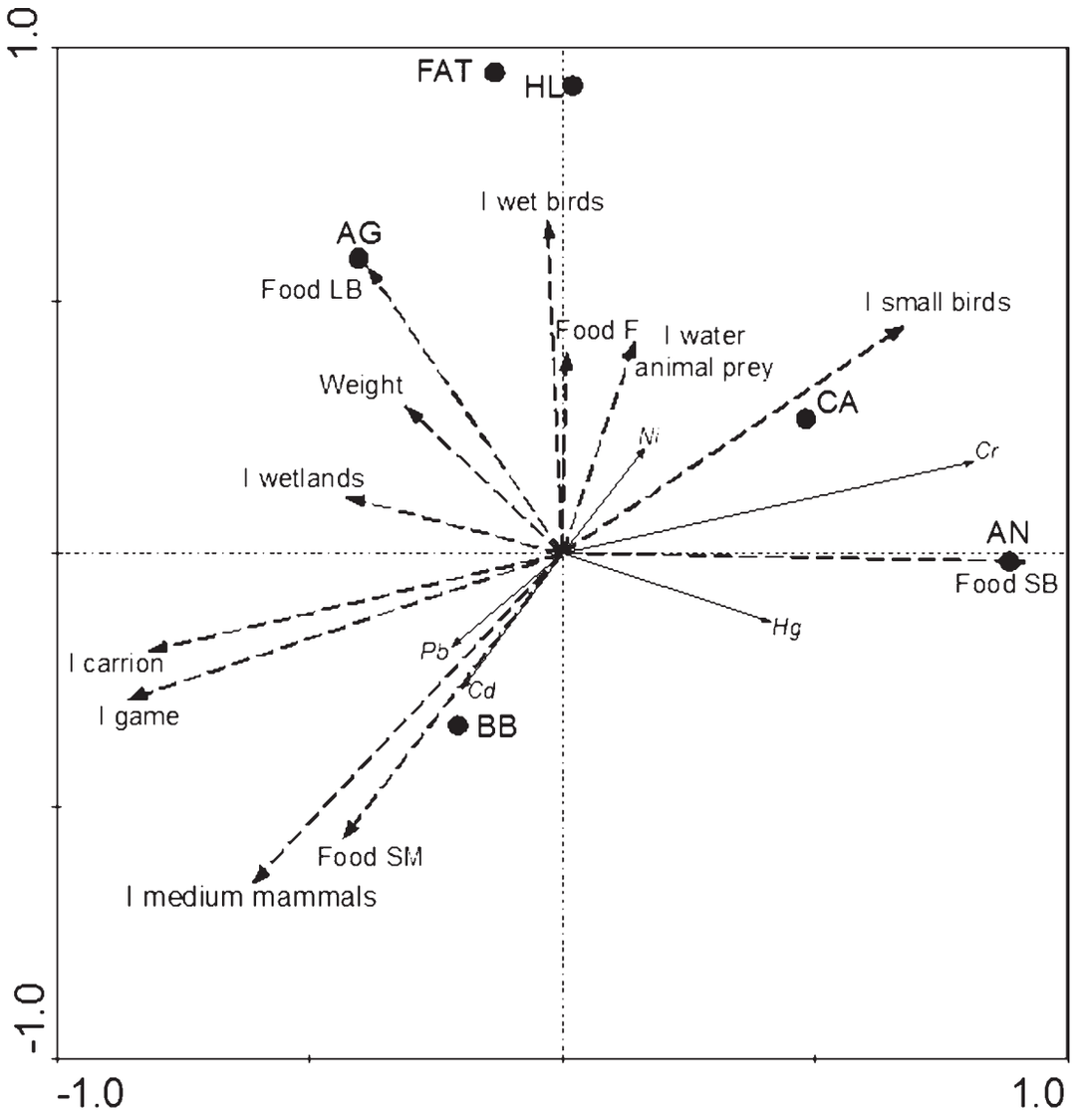

Fig.1. – Results of redundancy analysis (RDA) showing the effect of habitat parameters (dotted lines) on the concentration of heavy metals in the liver of raptors. Eigenvalues: axis 1 = 0.105; axis 2 = 0.029.
Abbreviations and scales used for analysis:
Species: HL = White-tailed eagle *Haliaeetus albicilla,* CA = Marsh harrier *Circus aeruginosus,* AG = Goshawk *Accipiter gentilis,* AN = Sparrowhawk *Accipiter nisus,* BB = Common buzzard *Buteo buteo,* FAT = Common kestrel *Falco tinnunculus.*
Food preferences: Food F = fish, Food SM = small mammals, Food SB = small birds, Food LB = large birds.
Importance of wetlands for foraging (I wetlands): 1 = marginal, 2 = medium, 3 = high, 4 = very high. Importance of game in food (I game): 0 = none, 1 = marginal, 2 = medium.
Importance of carrion of big and medium mammals in food (I carrion), importance of big wetland birds in food (I wet birds) and importance of small birds in food (I small birds): 0 = none, 1 = marginal, 2 = medium, 3 = high.
Importance of medium size mammals in food (I medium mammals): 0 = none, 1 = marginal, 2- medium. Importance of water animal prey (I water animal prey): 1 = small, 2 = medium, 3 = big.
Weight: bird mass [kg], according to CRAMP & SIMMONS (1980).

TABLE 2

Results of forward selection of habitat parameters. Variables with $P < 0.05$ were only included.

Variable	Lambda-A	F	P
Food preferences small birds (Food SB)	0.09	5.42	0.010

Pb hepatic concentrations and incidence of raptors with ≥ 6 mg/kg dw of lead

The RDA clearly shows that foraging on carrion and game causes elevated lead accumulation in the livers of some examined raptors, and this pattern was strongest for Buzzards (Fig. 1). It can be explained by changes in Buzzard hunting behaviour and the substitution of Common voles *Microtus arvalis* with other prey sources (JEDRZEJEWSKA & JEDRZEJEWSKI, 1998; VALKAMA et al., 2005; WUCZYNSKI, 2005; WIKAR et al., 2008). During the winter period, when availability of voles is limited because of snow cover, Buzzards may exploit wounded game or their carrion. Injured or dead game species with embedded or ingested lead may also become more easily available to raptors. On the whole, in our survey only three (9.7%) of 31 examined Buzzards revealed hepatic Pb concentrations ≥ 6 mg/kg dw. Other researchers (BATTAGLIA et al., 2005; ZACCARONI et al., 2008) found similar hepatic concentrations and proportions of birds with lead concentrations ≥ 6 mg/kg dw. Only one (3.2%) of the 31 Buzzards examined in our study had > 15 mg/kg of Pb dw in its liver. Similar limited numbers of Buzzards with such hepatic lead concentrations were reported in W Europe by other authors (PAIN & AMIARD-TRIQUET, 1993; PAIN et al., 1995).

Goshawk and Sparrowhawk generally do not scavenge frequently (ZIESEMER, 1983; NEWTON, 1986). Lead poisoning among free ranging Goshawks is rare indicating that these hawks seem to be at low risk to lead exposure (PAIN & AMIARD-TRIQUET, 1993). In Spain, CASTRO et al. (2011) described median lead concentration of 0.480 mg/kg dw for male and 0.798 mg/kg dw for female Goshawks, respectively. Goshawks examined in Germany (KENNTNER et al., 2003) accumulated, on average, 1.19 mg/kg ww of lead (4.76 mg/kg dw) while their median concentration was up to 0.133 mg/kg ww (0.532 mg/kg dw). The Pb levels measured in this study for Goshawks in East Poland were higher, suggesting that the more severe winters in East Poland as compared to Germany (WOS, 1999; BLAZEJCZYK, 2006) could increase scavenging behaviour in these birds.

In Sweden, 18 (15.5%) of 116 White-tailed eagle liver samples had Pb concentrations > 6 mg/kg dw with median concentrations of 0.601 mg/kg dw (range: 0.03 - 154.0 mg/kg dw) (HELANDER et al., 2009). None of the White-tailed eagles examined in this study had Pb hepatic concentrations > 6 mg/kg dw. This is consistent with the results of the performed RDA analysis illustrating the importance of fish in the overall prey as opposed to individuals that heavily exploit waterfowl and carrion. VAN RIJN et al. (2010) and NADJAFZADEH et al. (2013) proposed that White-tailed eagles respond to availability of fish. When the availability of fish sharply declines, eagles switch to waterfowl and carrion. The consumption of game carrion increases during autumn and winter and is correlated to a concomitant seasonal increase in the incidence of lead poisoning of eagles (HELANDER et al., 2009; NADJAFZADEH et al., 2013), an effect that we did not observe.

The RDA analysis also showed that the food preferences of Marsh harriers obviously allow them to avoid the accumulation of lead because their diet is mainly based on small or medium sized mammals, wetland birds, and carrion of fish (WITKOWSKI, 1989). Marsh harriers examined in this study accumulated less Pb in their livers

than Spanish raptors (with lead concentrations of 2.02 - 8.75 mg/kg dw; MATEO et al., 1999). One individual Marsh harrier found dead in France with an ingested lead bullet had hepatic Pb concentrations of 54.9 mg/kg dw (PAIN et al., 1993). Contrary to Western Europe (PAIN et al., 1995; PAIN et al., 1997; MATEO et al., 1999; MATEO, 2009), our study reveals that for Marsh harriers from Eastern Poland, hunted game not retrieved by hunters is not an important food source.

Cadmium levels and its sources

Cadmium concentrations in birds are generally reported to be the lowest in livers, while being intermediate in muscle tissue and highest in kidneys (GARCIA-FERNANDEZ et al., 1996; BATTAGLIA et al., 2005; BINKOWSKI et al., 2013). However, we assumed that using liver to monitor Cd exposure is still relevant because it accumulates about half of the total Cd body burden and Cd is stable in the liver (as liver is resistant to cadmium toxic effects; BURGAT, 1990; BATTAGLIA et al., 2005). The increasing concentration of Cd in Buzzard livers reported here could be directly related to their feeding preferences including voles. This is obvious from the results of the RDA analyses (Fig. 1) revealing a strong statistical effect of feeding on small mammals for elevated Cd levels.

Such a pattern can probably be attributed to the effect of the trophic preferences of common voles seized mainly on fields contaminated with fertilizers containing Cd (BEYER, 2000; FINNISH ENVIRONMENT INSTITUTE, 2000). Like other Eastern European countries, another significant source of Cd in East Poland is coal combustion (low emission from coal-burning stoves; PACYNA et al., 2011; SESHADRI et al., 2010). In our study, this threshold value > 3.0 mg/kg dw was exceeded only in a single Buzzard out of 31 specimens. The Cd concentrations in Buzzards from Northern Italy (median: 0.01 mg/kg dw, range: 0.017 - 2.02 mg/kg dw; BATTAGLIA et al., 2005), Portugal (mean 0.322 mg/kg dw, median: 0.184 mg/kg; CARNEIRO et al., 2014) and Spain (mean 0.364 mg/kg dw in males and 0.410 mg/kg dw in females, respectively; CASTRO et al. 2011) were much lower than in our study. Interestingly, Polish Buzzards previously examined by us accumulated clearly more Cd, namely on average 1.01 mg/kg dw Cd (10 specimens; KOMOSA et al., 2012).

Two other studies reported lower liver cadmium concentrations than our study. KENNTNER et al. (2003) examined specimens of Goshawk from Germany, which had overall mean cadmium concentrations of 0.06 mg/kg ww and mean liver concentrations of 0.24 mg/kg dw while CASTRO et al. (2011) reported a median hepatic Cd concentration of 0.146 mg/kg dw (male) and 0.154 mg/kg dw (females), respectively. Interestingly, in Sweden, a similar hepatic cadmium level (0.80 mg/kg dw) to Goshawks from our studies was found for Peregrines (*Falco peregrinus*) feeding on birds of bigger sizes (EK et al., 2004) like Goshawks do. FALANDYSZ et al. (2001) reported a two-fold lower Cd level (0.15 mg/kg dw) in livers of ten Baltic White-tailed eagles than our measurements for the same species (Table 1).

Mercury levels and its sources

The RDA showed that consumption of small birds such as passerines can be a source of mercury contamination (Fig. 1). This is so because higher concentrations of mercury are strongly dependent on the extent of feeding on passerines or grains dressed with Hg-based fungicides. Such a correlation was strongest for Sparrowhawks. Earlier reports (JOHNELS et al., 1979; SOLONEN & LODENIUS, 1984) showed that granivorous birds can be the main cause of Hg accumulation in Sparrowhawks because they constitute approximately 1/5 of the prey consumed during the breeding season. Such contamination as reported here may indicate a return to dressed seed usage in Poland or neighbouring countries. For Buzzards examined in Spain, median hepatic mercury concentration

amounted to 1.96 mg/kg, both for males and females (CASTRO et al., 2011), which was three times more than the concentrations found in the current study. Also livers of Buzzards from Czech Republic showed a median Hg level of 2.61 mg/kg dw (HOUSEROVA et al., 2005) which was 3-fold higher than that determined in our survey. For Spanish Goshawks, CASTRO et al. (2011) measured median Hg hepatic levels of 0.236 mg/kg dw and 0.182 mg/kg dw, respectively, for males and females, which are far below our measurements. These estimates may reflect a preference for seizing avian prey of smaller size, including passerines feeding on illegally dressed grain. This may also explain why maximal Hg levels were 8-fold higher in males than females (CASTRO et al., 2011). In our study, the strong correlation between mercury and the consumption of small birds in the RDA analyses also implies that consumption of bigger avian prey by Goshawks allows them to avoid the accumulation of large amounts of mercury in the livers, in contrast to the Sparrowhawks specialized in catching small birds (NEWTON, 1986). Goshawk individuals examined in Germany had very similar levels of hepatic mercury to those in the current study, with median values of 0.069 mg/kg ww (about 0.276 mg/kg dw; KENNTNER et al., 2003).

Many researchers have pointed out the disastrous state of waterbodies and the sea and the resulting contamination of water prey as a mercury source in raptors (FALANDYSZ et al., 2001; KALISINSKA et al., 2014). However, during the last years, water quality has very much improved. When examining livers of eight White-tailed eagles from south Baltic coasts, FALANDYSZ et al. (2001), found a mean value of 5.8 mg/kg Hg which was 6-fold higher than our findings (Table 1). Recent analysis of White-tailed eagles (KALISINSKA et al., 2014) showed that mercury contamination in the Polish Baltic region has been reduced as a result of a decrease of heavy metal emissions. This was confirmed by studies on other biota from the same region (GUSEV, 2013; NYBERG et al., 2013). As demonstrated by our RDA analysis, water animals as prey were also not a significant source of contamination for Polish raptors.

Chromium and nickel

For Marsh harrier, kestrel, Buzzard and Sparrowhawk from south Italy, the mean hepatic concentrations of chromium never exceeded 0.40 mg/kg dw (ZACCARONI et al., 2008). In the same study, Tawny owls (*Strix aluco*), which are known to prey frequently on passerines, had mean hepatic concentrations slightly exceeding 0.30 mg/kg dw. A similar value was determined from another study from north Italy on the Little owl (*Athene noctua*) for which a concentration of 0.29 mg/kg ww (i.e. about 1.16 mg/kg dw.) was determined. Both estimates from Italy are very similar to the levels obtained for Sparrowhawks in this study. An important diet component of Italian Little owls is passerines (ZACCARONI et al. 2003). This finding fits our data as the RDA analysis showed that consumption of passerines is not only correlated to elevated concentrations of mercury but also of chromium (Fig. 1). FALANDYSZ et al. (2001) measured mean Cr concentration of 0.087 mg/kg dw in three specimens of White-tailed eagles from the south Baltic, which is considerably less than our results from inland White-tailed eagles. MANNING et al. (2000) reported an even lower Cr hepatic value for a single Australian White-bellied sea-eagle (*Haliaeetus leucogaster*) of 0.04 mg/kg ww (i.e. about 0.16 mg/kg dw). In contrast, for White-backed vulture (*Pseudogyps africanus*) (n=5 specimens) from the Republic of South Africa, VAN WYK et al. (2001) estimated on average 19.57 mg/kg dw of hepatic Cr, which is higher than what we measured.

Sparrowhawks and Goshawk from our study, being specialised in seizing birds, accumulated almost similar average levels of Ni (0.3 mg/kg dw). A single specimen of Peregrine (*Falco peregrinus*) also preying on birds from NW Poland had hepatic Ni concentration of only 0.107 mg/kg dw (KALISINSKA et al., 2008). FALANDYSZ et al. (2001) showed that livers of

White-tailed eagles had 56-fold higher hepatic nickel concentrations (13.0 mg/kg dw) than our findings. It seems that White-tailed eagles from East Poland have less opportunities to prey in highly mercury- and nickel-contaminated areas than raptors from the south Baltic Sea coast.

CONCLUSIONS

We could show that food preferences of raptors can strongly influence the load of heavy metals as is, for example, evidenced by the elevated concentrations of Hg and Cr in Sparrowhawks. This can be explained by their preying on small birds. Similarly, through scavenging on game and its carrion, Buzzards are more exposed to Pb, probably originating from lead bullets of hunters. That the impact of human activities on heavy metal accumulation in raptors can also be reduced, is illustrated by our data from White eagles preying on fish, where the amount of Hg contamination in aquatic habitats has decreased significantly in the last years.

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A quadratic approach to allometry yields promising results for the study of growth

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ABSTRACT. Julian HUXLEY (1924) came to the conclusion that intra-specific growth usually follows a sequence of power curves. So HUXLEY claimed that during growth sudden changes in the growth rate can occur. The restudy of his material, however, reveals that his observations closely follow single quadratic curves. As a result the intra-specific allometry studied by HUXLEY is comparable to ontogenetic allometry. The quadratic factor of the quadratic equations obtained, represents the growth rate; it shows the constant increase (positive factor) or decrease (minus factor) of one of the measurements for a constant increase in the other measurement with which it is compared. The quadratic factor explains the entire growth process and is the same for the smaller (younger) and larger (older) specimens. It could probably permit the prediction of the shape of larger and/or smaller animals not yet found, or give a clue to some evolutionary changes. By using the quadratic parabola there is no need to postulate "sudden changes in the growth curve" and so it appears that HUXLEY's power curve can be abandoned.

KEY words: allometry, Huxley, parabolic curve, quadratic equation, shore crab, stag beetle.

INTRODUCTION

HUXLEY's (1924) assumption was, when he compared the measurements of body part y with body part x, that for a theoretical, small amount of growth, there is a constant ratio between the two growth rates

$$dy/dx = \text{constant } k \quad (1)$$

This resulted in the formula of allometric growth:

$$y = bx^k \quad (2)$$

"b" and "k" being constant factors. This exponential formula can also be written as:

$$\log y = \log b + k \log x \quad (3)$$

As a result the curvilinear relationship (2) is linearized when the data are plotted onto a log-log scale; the slope of that line is represented by the power factor "k" (known as the allometric coefficient) and log b is the intercept of the line on the y-axis.

The study of growth has been, thereafter, greatly influenced by Huxley's proposal, although doubts have also been expressed. The results on log-log graph paper often show not a single straight line, but two-three consecutive straight lines. These observations have been explained by proposing 'sudden changes" in the allometric constant k. These "sudden changes" have cast some doubt on the allometric formula and several other formulas have been proposed (a review is found in ZEGER & HARLOW, 1987). Recent publications on that matter are e.g. STERN & EMLEN (1999); GAYON (2000); KNELL et al. (2004); SHINGLETON (2010) and PACKARD (2012).

The term allometry was introduced by HUXLEY & TEISSIER (1936). It designates the changes in relative dimensions of parts of an organism that are correlated with changes in shape and overall size (LEVINTON, 1988 in GAYON, 2000).

PACKARD (2012) restudied HUXLEY's measurements of *Uca pugnax* (SMITH, 1870) and presented not only a two-parameter power function but also a three-parameter power function; this three-parameter model "is better than the two-parameter model for describing

the observations". In a remark on that article, GERAERT (2013) suggested that a quadratic parabolic curve is as good as or even better than a power function to explain the observations.

GERAERT (2004), studying ontogenetic allometry, has shown that there is a constant change in the relationship (and not a constant relationship) between a small amount of growth of body part y compared to that of body part x; mathematically speaking, "the second difference" is constant. That second difference is the growth rate and is present in the quadratic factor of a quadratic equation; the other factors in that equation have no biological meaning but are necessary to position the quadratic curve in a diagram.

GERAERT (2004) followed growth data from the new-born stage to the adult. HUXLEY (1924, 1932) based his assumptions mainly on the variation found in adults, not only on the males of the fiddler crab, but also on the males and females of the shore crab (*Carcinus maenas*) and on male mandibles in three species of stag-beetles (Lucanidae). The very large variation observed in these adults was interpreted by HUXLEY (1924, 1932) also as "growth". In this study, an attempt is made to see if a quadratic parabola can also be used to describe variation in adults, called "comparative" growth in GERAERT (2013), and "static" and "intra-specific" allometry in GOULD (1966) and GAYON (2000).

RESULTS

Carcinus maenas L. (Fig. 1)

HUXLEY & RICHARDS (1931) studied the increase of width of the abdomen in comparison with the increase of carapace length; the measurements were split into three categories: "unsexables", females and males. HUXLEY

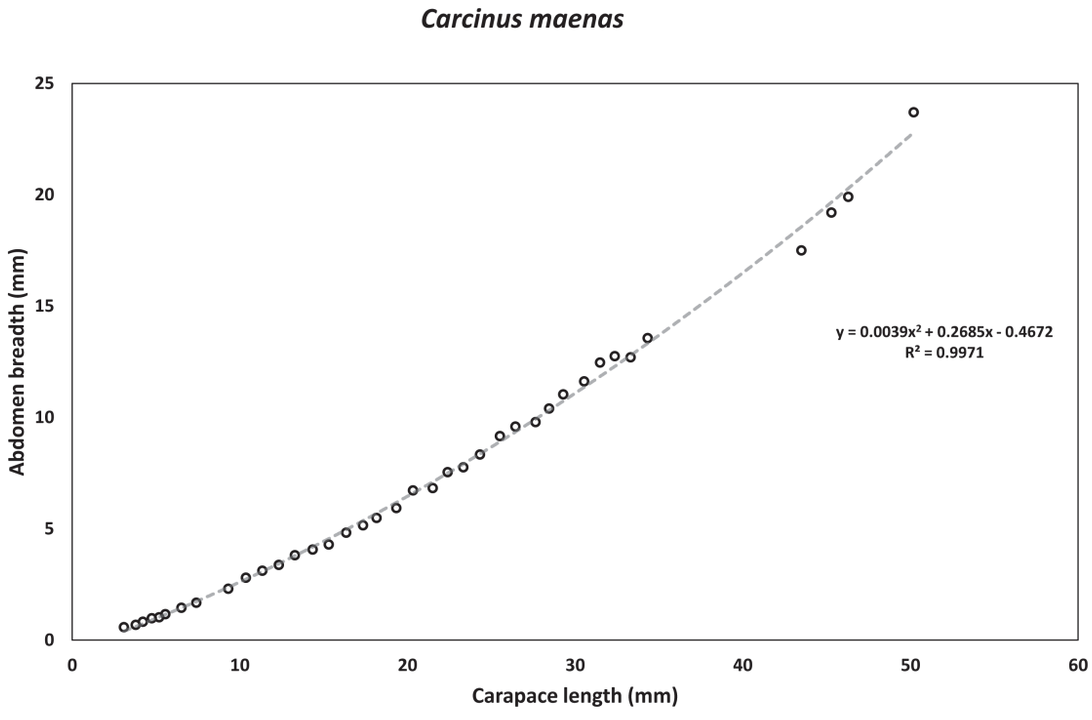

Fig. 1. – Comparison of carapace length to abdomen breadth in *Carcinus maenas*. The measurements given in HUXLEY (1932) are represented on a double arithmetic scale (and not on a log-log scale); the open circles are the measurements for the unsexed specimens and for the adult females of the shore crab. The calculated quadratic parabola is added.

(1932) gave the measurements for the unsexables and for the females; he used "abdomen-breadth" as Y and "carapace-length" as X (both in mm). The quadratic equation linking both is:

$Y = 0.0039\ X^2 + 0.2685\ X - 0.467$ $(R^2 = 0.997)$

Per 10 mm increase in carapace length the abdomen breadth shows a constant secondary increase of 0.78 mm (this is twice the quadratic factor). The differences between the observed Y-values and the calculated Y-values are small for whatever X-value is considered (Table 1).

$\sum (Y \text{ calculated} - Y \text{ observed})^2 = 3.7 \text{ mm}^2$

The mean difference between Y calculated and Y observed is approximately 0.1 mm; this difference varies from zero to 0.9 mm.

Cyclommatus tarandus (THUNBERG , 1806)

(Fig. 2)

HUXLEY (1927, 1932) studied the increase of the mandibles in several species of the Lucanidae, the stag beetles; he used the measurements of DUDICH (1923) for *Cyclommatus tarandus*. In this case Y = mandible length in mm and X = body length + mandible length also in mm. The quadratic equation linking both is:

$Y = -0.0011\ X^2 + 0.71\ X - 11.41$ $(R^2 = 0.997)$

Per 10 mm increase in the total length X there is a constant secondary decrease of 0.22 mm in the mandible length (this is twice the quadratic factor); this small quadratic factor indicates that an almost straight line is observed (This may be largely due to the fact that measurement Y is included in X). The differences between the observed Y-values and the calculated Y-values are small for whatever X-value is considered (Table 1).

$\sum (Y \text{ calculated} - Y \text{ observed})^2 = 5.35 \text{ mm}^2$

The mean difference between Y calculated and Y measured is approximately 0.44 mm; this difference varies from zero to 1.3 mm.

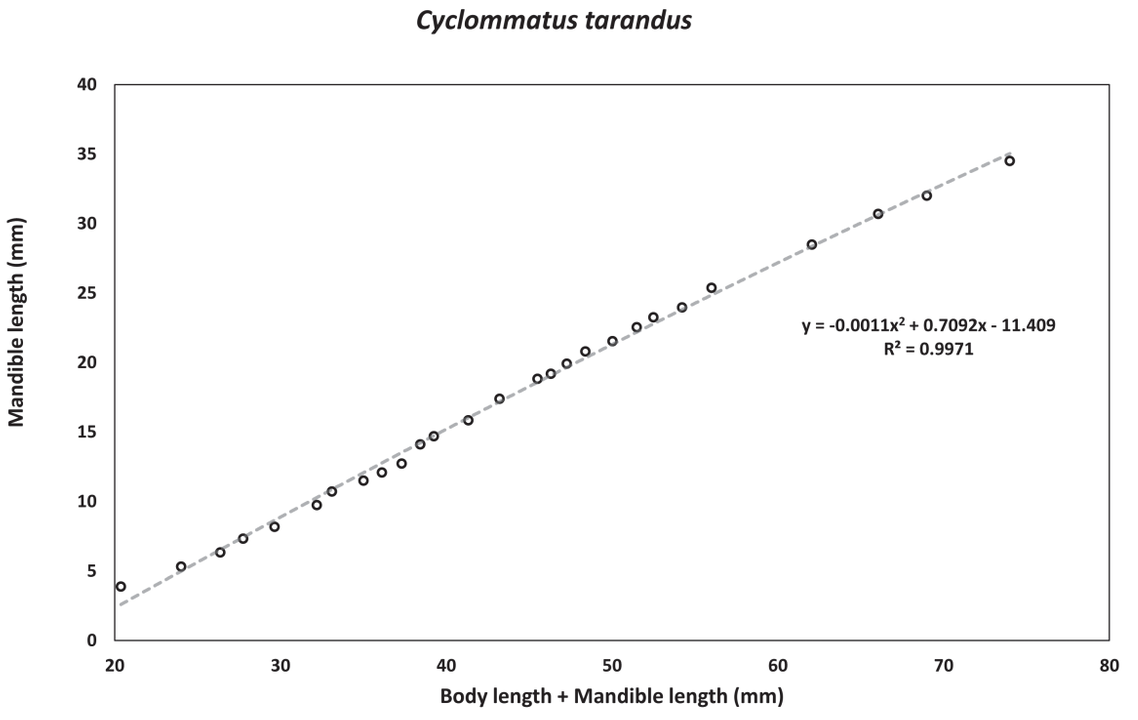

Fig. 2. – Comparison of total length (= body length + mandible length) to mandible length in *Cyclommatus tarandus*. The measurements given in HUXLEY (1932) are represented on a double arithmetic scale (and not on a log-log scale); the open circles are the measurements for the males of this stag beetle. The calculated quadratic parabola is added.

TABLE 1

Calculated values for *Carcinus maenas* and *Cyclommatus tarandus* based on the measurements given in HUXLEY (1932). The calculated values are obtained by using the quadratic equation shown in the text; the Y-values are calculated by a constant increase of the X-values with 10 mm. As a result the constant second differences in the Y-values are obtained. X and Y are explained in the text.

CARCINUS MAENAS				*CYCLOMMATUS TARANDUS*			
X-value in mm	Y-value calculated	Increase in Y-values	Second difference	X-value in mm	Y-value calculated	Increase in Y-values	Second differences
10	2.606			20	2.336		
		3.850				6.543	
20	6.456		0.78	30	8.879		-0.22
		4.627				6.322	
30	11.083		0.78	40	15.201		-0.22
		5.403				6.102	
40	16.487		0.78	50	21.303		-0.22
		6.180				5.882	
50	22.667			60	27.186		

DISCUSSION

The allometric equation (2) (3) needs two factors ("b" and "k") to describe the relationship between the measurements X and Y. The meaning of each one of them has been a debate during many years and is summarized in GAYON (2000); no final conclusion is reached. The quadratic equation has only one factor that shows the constant increase (positive factor) or decrease (minus factor) of one of the measurements for a constant increase in the other measurement with which it is compared.

HUXLEY (1932) did not find the single straight line (3) needed to support his theory in the two cases restudied here (and in the fiddler crab restudied in GERAERT, 2013). For the shore crab the logarithmic plotting showed a kink in the observations as well for females as for males, so a different growth coefficient was observed for young females (males) and older females (males). HUXLEY (1932) gave several k-values (added on Fig. 3) that he experimentally derived from his figure, moreover the constant "b" has not been given. Therefore it is not possible to compare his (several) equations with the single quadratic equation obtained. The straight lines found by HUXLEY (1932) in his log-log diagram (Fig. 3) can be interpreted as mathematical accidents; on the other hand in Fig. 1 one can suggest another three consecutive straight lines: these are mathematical accidents as well in this arithmetic diagram.

For the stag beetles HUXLEY (1932) found that all curves inflect at large absolute sizes; for the smaller animals of *Cyclommatus tarandus* he gave a k-value of 1.97 and a b-value of "just over 0.01". (Fig. 4). So, it is not relevant to compare his curve restricted to the smaller animals with the quadratic one presented here, that includes all the measurements.

Nevertheless HUXLEY continued propagating the power curve for describing growth. His proposal has been generally accepted, as for example in recent times by KNELL et al. (2004) for the stag beetle; SHINGLETON (2010) for the fiddler crab. On the other hand the quadratic curve closely follows all the observations including those relating to the smallest and the largest animals; the single quadratic factor explains the entire process.

CHAMPY (1924), cited by GAYON (2000), argued that the relative growth process was adequately described by a parabolic curve of

the shape $V = at^2$; this curve is a special case of a power curve and is different from the curve proposed here. TEISSIER (1931), cited by GAYON (2000), observed that CHAMPY's law was indeed a good approximation for some insects. MARTIN (1960), KIDWELL & HOWARD (1970) and WALKER & KOWALSKI (1971) using curve-fitting programs observed that a parabolic curve gave the best approximation for their measurements on growth; every one of these authors stressed that this discovery was arbitrary and had no biological meaning. Apparently no one thereafter used or mentioned the quadratic equation (ZEGER & HARLOW, 1987).

CONCLUSIONS

1. The quadratic equation explaining ontogenetic allometry (GERAERT, 2004) explains (in the cases studied) intra-specific allometry as well.
2. HUXLEY's (1924) proposal to use a power curve to explain intraspecific allometry seems no longer acceptable. By using the quadratic curve there is no need for such explanations as "sudden changes in the growth factor" or "the curve inflects at large absolute size"; there is one and only one factor that describes relative growth and that is the quadratic factor of the quadratic equation.

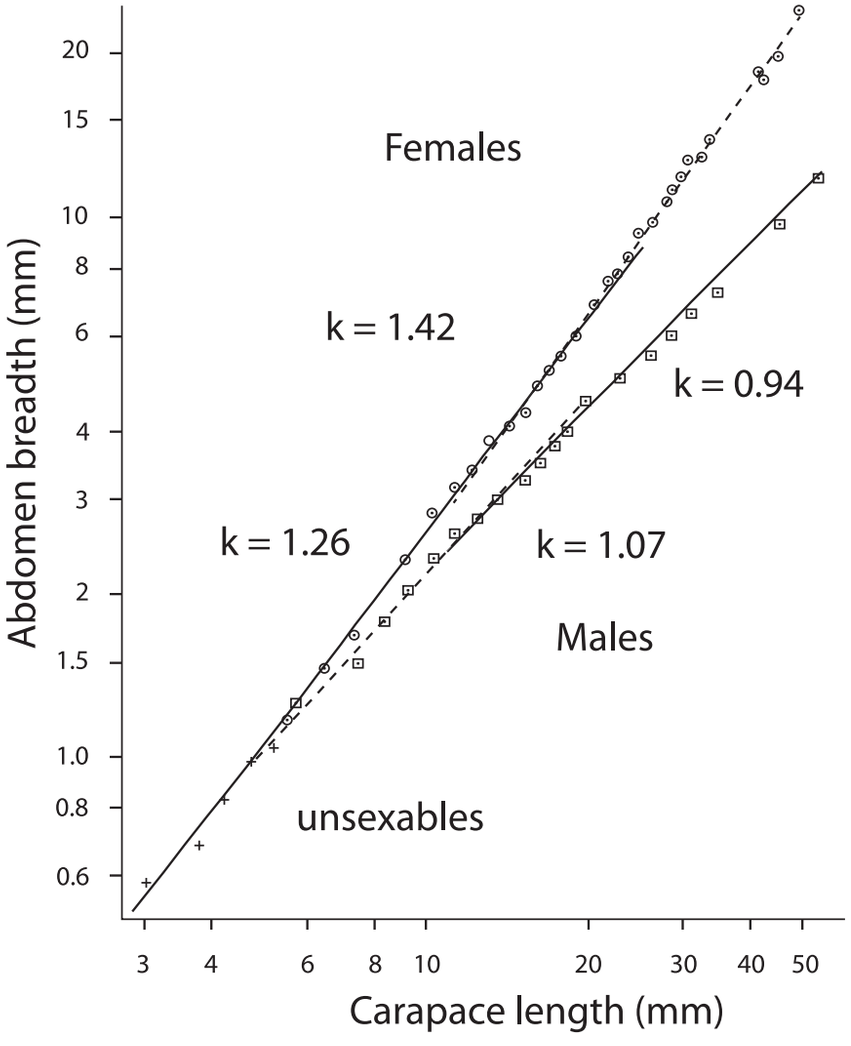

Fig. 3. – Figure taken from HUXLEY (1932) with the following explanation: "Increase of width of abdomen with increase of carapace length in the shore crab, *Carcinus maenas*: logarithmic plotting". The signs for unsexables, males and females are explained on the graph; the growth coefficients given by HUXLEY (1932) were also added.

Presumptions

3. The use of a quadratic equation in a case of intra-specific allometry could allow prediction of the shape of larger and/or smaller specimens not yet observed.

4. It can be assumed that changes in the growth factor(s) do now-and-then occur and have occurred in the past; such shifts could perhaps explain some evolutionary changes.

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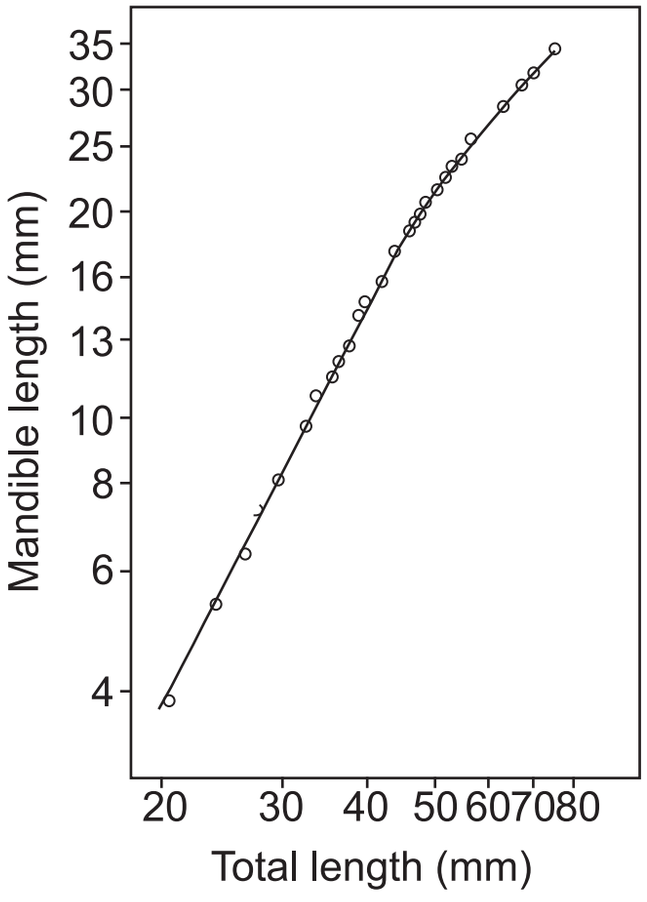

Fig. 4. – Figure taken from HUXLEY (1932) with the following explanation: "Relative growth of male mandibles in three species of stag-beetles (Lucanidae)". The results for *Lucanus cervus* and *L. lunifer* were, however, omitted. The explanation for *Cyclommatus tarandus* reads as follows: "Total length is true total length (= body length + mandible length). The curve inflects at large absolute size; for the remainder of the curve k is about 2.0".

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Trophic ecology of the seagrass-inhabiting footballer demoiselle *Chrysiptera annulata* (Peters, 1855); comparison with three other reef-associated damselfishes

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ABSTRACT. Many damselfishes (Pomacentridae) are herbivorous or omnivorous with an important contribution from different kinds of algae in their diet. They display different levels of territoriality and farming behavior, from almost non territorial to monoculture farmers. In addition, a few species inhabit seagrass meadows but, presently, none can be considered as seagrass-eating specialists. The footballer demoiselle, *Chrysiptera annulata*, is found in the seagrass meadows on the reef flat of the Great Reef of Toliara (Madagascar, Mozambique Channel). In the light of this unusual habitat for a pomacentrid, this study aimed to answer three questions: 1) What is the diet of *C. annulata*? 2) Do the resources supporting this diet include seagrass? 3) Does its trophic niche overlap those of other sympatric damselfishes (*Pomacentrus trilineatus, Chrysiptera unimaculata* and *Plectroglyphidodon lacrymatus*) living in close association with macrophytes or eating algae? Stomach content examination and stable isotope analysis showed that the footballer demoiselle is not a seagrass consumer but is an omnivorous/herbivorous species heavily relying on algal resources and small invertebrates. SIAR, a stable isotope mixing model, indicated it assimilated large amounts of turf algae, and various benthic or planktonic invertebrates in lower proportions. SIBER metrics revealed that the isotopic niche of the footballer demoiselle partly overlaps that of its congener, *C. unimaculata*, but not those of *P. trilineatus* and *P. lacrymatus*. Trophic strategies of *C. annulata* differed both from farming species such as *P. lacrymatus* and from less territorial herbivores such as *P. trilineatus*. Its seagrass meadow habitat on the Great Reef of Toliara allows the conquest of an unusual habitat for damselfishes and could limit competition with *C. unimaculata*, a species displaying the same territorial behavior and the same isotopic niche but living on the reef itself.

KEY WORDS: herbivory, stable isotopes, coral reef, seagrass, trophic niches, Western Indian Ocean, SIAR, SIBER.

INTRODUCTION

The Great Reef of Toliara (SW Madagascar, Mozambique Channel) was once one of the most diverse coral reefs in the Western Indian Ocean (WIO) (Pichon, 1978). Unfortunately, recent observations highlight drastic changes in its biodiversity (Harris et al., 2008; Andréfouët et al., 2013). Coral cover has drastically decreased on the reef flat with the disappearance of branched *Acropora* spp. (Harris et al., 2008) whereas macroalgae cover has significantly increased between 1978 (Pichon, 1978) and 2008 (Harris et al., 2008). Concomitantly, fish communities have also changed as they were previously dominated by carnivores (Harmelin-Vivien, 1979) and are now dominated by herbivores and omnivores (Harris et al., 2008).

Since large herbivore biomass is strongly affected by overfishing on the Great Reef of Toliara, most of the remaining herbivores are small species such as damselfishes (Pomacentridae) (Harris et al., 2008; Frédérich, pers. obs). Herbivorous fishes display a high trophic diversity due to differences in ingested species, nutritional strategies, eco-morphological adaptations, contribution from other food sources or foraging patterns (Choat et al., 2004; Fox & Bellwood, 2013; Bellwood et al., 2014).

For example, some species may be classified as "roving herbivores" (or foragers), sometimes forming schools, having no feeding territories (or very large territories) and/or exploiting diverse habitats for feeding (FRÉDÉRICH et al., 2009; GULLSTRÖM et al., 2011; PLASS-JOHNSON et al., 2013). Others may be solitary, territorial and restricted to a particular area for feeding (FISHELSON, 1998; CECCARELLI et al., 2005). An extraordinary adaptation of some damselfishes is to garden these small territories, determining the algal community and abundance within the small area they defend against conspecifics and other herbivores (CECCARELLI et al., 2005). The occurrence of multiple herbivorous damselfish species raises the question of the diversification and overlap of trophic niches among these species.

The footballer demoiselle, *Chrysiptera annulata* (PETERS, 1855), is one of the smallest pomacentrids (FISHELSON, 1998). It is a Western Indian Ocean (WIO) species, inhabiting reef flats of the southern Malagasy coast (GILLIBRAND et al., 2007) but also other islands of the Mozambique Channel (WICKEL et al., 2014) as well as the East African coast (GARPE & ÖHMAN, 2003) and the Red Sea (KHALAF, 2004). *Chrysiptera annulata* is a solitary species, defending, against conspecifics, small areas (< 1m^2) constituted by a small coral boulder often bearing macroalgae such as *Padina* spp., *Sargassum* spp or *Turbinaria* spp. (FISHELSON, 1998). On the Great Reef of Toliara, *C. annulata* is almost restricted to subtidal seagrass meadows found on the reef flat. This particular habitat and territorial behaviour raise the question about the contribution of plant materials to its diet and about its potential consumption of seagrass. Seagrass consumption and/or assimilation by animals were traditionally considered as negligible to very low due to their low palatability (CEBRIAN, 2002). Nevertheless, seagrass herbivory is highly variable and its level differs in tropical and in temperate and high latitude seagrass meadows (HECK & VALENTINE, 2006). Indeed, tropical seagrass palatability is probably comparable to many macroalgae (KLUMPP et al., 1993) and more consumers than assumed before seem to depend on seagrasses for their food (VONK et al., 2008). Among reef fishes, seagrass consumption is unambiguous for some scarid species (GULLSTRÖM et al., 2011) but it is not demonstrated for pomacentrids living in seagrass beds (e.g. NAKAMURA et al., 2003).

Stable isotopes have a long history as a powerful method allowing the assessment of trophic ecology of fishes and other animals (LAYMAN et al., 2012). The method relies upon the fact that the isotopic composition of a consumer is the weighted average of the isotopic compositions of its food sources, modified by the net isotopic fractionation between diet and animal tissues (i.e. the change of the isotopic composition of food sources during food processing, assimilation and metabolism). A more recent application of stable isotopes analysis is to characterize the trophic niche using the isotopic variability inside communities, species or populations (LAYMAN et al., 2007; NEWSOME et al., 2007). This so-called "isotopic niche" is generally used as a proxy of the trophic niche.

Here, we used measurements of carbon and nitrogen isotopic ratios and stomach content analysis to investigate trophic ecology of *C. annulata* in the seagrass meadows from the Great Reef of Toliara. Our first objective was to determine whether this damselfish was herbivorous and whether the consumption of seagrass could represent an unexpected specialization of this species. Our second goal was to test the hypothesis that different herbivorous damselfishes may have different isotopic niches in relation to differences in their habitat, feeding strategy or behaviour. We have therefore compared the isotopic niche of the footballer demoiselle to those of three other damselfishes from the Toliara great reef: (1) *Pomacentrus trilineatus* Cuvier 1830, an omnivorous species with significant contribution from algae in its diet (FRÉDÉRICH et al., 2009), often living in small aggregations on the coral reef flat and considered as a less territorial species (gregarious polydomous *sensu* FISHELSON, 1998); (2) *Chrysiptera unimaculata* (CUVIER, 1830), an algivorous species also living on the coral reef flat (FRÉDÉRICH et al., 2009) and being

territorial but not considered as a strict farmer in this location (FRÉDÉRICH, personal observation); and (3) *Plectroglyphidodon lacrymatus* (QUOY & GAYMARD, 1825), a well-known territorial farming species (JONES et al., 2006) living in the lagoon (FRÉDÉRICH et al., 2009).

MATERIALS AND METHODS

Chrysiptera annulata (n = 35) were sampled by scuba diving in May 2011 on the flat of the Great Reef of Toliara (6 m depth at high tide, less than 3 m at low tide). Thirty individuals were sampled in monospecific *Halophila stipulacea* seagrass patches or *Syringodium isoetifolium*-dominated polyspecific patches (secondary species: *Cymodocea rotundata* and *Halodule uninervis*). Five were collected close to this area in a site with coral boulders colonized by brown algae (*Sargassum* spp.) but without seagrass meadows. Fish were always closely associated to little rocks or coral rubbles (territories less than 1m^2 and almost equal between individual) bearing sometimes large erect brown macroalgae (*Sargassum* spp. or *Turbinaria* sp. for example), foliose macroalgae (e.g. *Padina* spp.) and smaller erect (*Hypnea* spp.) and always turf macroalgae (*Polysiphonia* spp.), growing on the boulder or as epiphytes of large erect algae. Each territory was defended by one individual and occupied by 1 to 4 individuals. All *C. annulata* specimens were collected between 9:00 and 12:00 am after being anesthetized with clove oil. Twenty-nine specimens of *Pomacentrus trilineatus* were also collected from two sites distant a few hundred metres and close to the area where we caught *C. annulata* specimens. Specimens of *Pomacentrus trilineatus* were caught along the slope of small water basins locally called "vasques", which constitute deeper water areas within the reef flat and are colonized by living corals but were devoid of seagrass patches.

After their capture, fishes were brought to the surface, killed by immersion in MS-222 and placed on ice before return to the laboratory. In the laboratory, standard length (SL) was measured to the nearest mm with a Vernier calliper and fishes were dissected to collect samples (approx. 2 cm^3) of lateral muscle tissue for stable isotope analysis. The entire digestive tract was then removed and conserved in 70% ethanol for stomach content analysis.

For each individual, potential food sources (i.e. seagrass, erect macroalgae, their respective epiphytes and other macroalgae attached to rocks and rubbles) were also sampled for isotopic analysis. Potential animal food sources such as zooplankton and small benthic invertebrates (e.g., isopods and harpacticoid copepods) were taken from the fish collection site according to the methodology described in FRÉDÉRICH et al. (2009). Suspended particulate organic matter was collected by filtering five litres per sample of pre-sieved (250 μm) seawater from the collection site on a glass fibre filter (GF/C, Whatman).

Stomach content analysis

Stomachs were opened and their contents visually examined using a Wild M10 binocular microscope. First, the methodology of WILSON & BELLWOOD (1997) was used in order to quantify food items. Stomach contents were spread over a Petri dish, covering a 15 × 15 square grid (5 × 5 mm) placed underneath. For each of 50 randomly marked grid quadrats, the dominant item (by area) was recorded, along with any other material present in the quadrat. The data were condensed into seven categories: turf algae, erect macroalgae, seagrass, sessile benthic invertebrates, vagile benthic invertebrates, zooplankton and undetermined (i.e. item impossible to identify). Then, we calculated the contribution of each category within stomach content, and values for each category were expressed as the percent of quadrats in which that category was dominant. Finally, we also calculated the percentage of occurrence of each of the seven food categories (HYSLOP, 1980).

Stable isotope analysis

Samples of lateral muscle tissue from *C. annulata* and *P. trilineatus* and potential

food sources were dehydrated for 24h at 50°C before being ground into an homogenous powder. After grinding, samples containing carbonates (calcified algae, zoobenthos and zooplankton) were placed for 24 h under a glass bell with fuming HCl (37%) (Merck, for analysis quality) in order to eliminate calcareous material. Carbon and nitrogen stable isotope ratios of fishes were analysed on an IR-MS (Isotope Ratio Mass Spectrometer, VG Optima, Micromass, UK) coupled to an N-C-S elemental analyser (NA1500, Carlo Erba, Italy). Isotopic ratios of food sources were measured on IR-MS (Isoprime 100, Isoprime, UK) coupled with 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-N1 ($\delta^{15}N = +0.4 \pm 0.2‰$) and IAEA CH-6 (sucrose) ($\delta^{13}C = -10.4 \pm 0.2‰$). Routine measurements were precise to within 0.3‰ for both $\delta^{13}C$ and $\delta^{15}N$.

Data treatment and statistics

The stable isotope mixing model SIAR (Stable Isotope Analysis in R) was used to estimate the relative contribution of different prey species (isotopic sources) to the diet of footballer demoiselle (PARNELL et al., 2010). SIAR (Version 4.2) was fitted in R (R 3.1.3, R Development Core Team 2008) including isotopic compositions of each consumer, isotopic compositions of food sources 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). Model was run with the following potential food sources: zooplankton, zoobenthos and turf algae. For carbon, we have used a unique TEF factor of 1.6 ± 0.5 ‰ which was already used by DROMARD et al. (2015) and is in the range of generally observed TEF for ^{13}C in omnivorous fishes (PLASS-JOHNSON et al., 2013). TEFs for nitrogen were adapted according to food type potentially assimilated by the fish. For algal sources, we have used a TEF of 5.1 ± 0.6 ‰ (adapted from MILL et al., 2007), which is observed for farmer damselfishes such as *Stegastes* spp. (HATA & UMEZAWA, 2011) or herbivorous scarids and acanthurids (MILL et al., 2007). For animal sources, we have used a TEF for nitrogen of 2.3 ± 0.5 ‰, calculated by DROMARD et al. (2013) using equations of CAUT et al. (2010). Model was run with 10^6 iterations and burn-in size was set as 10^5. Model solutions were presented using credibility intervals of probability density function distributions (PARNELL et al., 2010).

Isotopic niche parameters were computed using SIBER (Stable Isotope Bayesian Ellipses in R; JACKSON et al., 2011), a part of the R package SIAR (see above). Isotopic data used for *C. unimaculata* and *Pl. lacrymatus* come from FRÉDÉRICH et al. (2009). They were measured on fish taken close to the present study location (less than 500 m) in 2008, measured in the same laboratory using continuous flow EA-IRMS. SIBER was used to generate bivariate standard ellipses that represent core isotopic niches of consumers. Areas of the ellipses associated to 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 probability density function distributions.

Relationship between stomach contents and fish size were tested through linear regressions. Differences among stable isotopic compositions of food sources and consumers were tested using a non-parametric Kruskal-Wallis test, because conditions for parametric approach were not respected for all groups. Dunn's Multiple Comparison Tests were used to assess pairwise differences when Kruskal-Wallis revealed statistically significant effects. A Mann-Whitney U test was used to test difference between the stable isotopes composition of *P. trilineatus* in their two sampling locations. All test results were considered as significant when p was ≤ 0.05. Statistical analyses were conducted using Prism 5.04 (GraphPad Software, La Jolla, U.S.A.).

RESULTS

Stomach contents of *Chrysiptera annulata*

Stomach contents are composed of diverse fractions but are dominated by filamentous turf algae (Table 1, Fig. 1). 97% of fish showed turf algae in their stomach content (Table 1) and this item accounted for more than 55 % of the stomach content in the great majority of studied specimens (Fig. 1). The relative amount of turf algae was the only item that was positively correlated to fish size (R^2= 0.20; p= 0.01; Fig. 1). Seagrass fragments were observed in the stomach of only two specimens (6% frequency of occurrence), despite the fact that 30 individuals out of 35 were caught on small territories at the border of or within seagrass patches (Table 1). The amount of erect macroalgae found in stomach contents was generally negligible. Zooplankton contributed importantly to the diet of smaller specimens. This contribution tended to decrease as size increased and was equal to zero from a size of 50 mm SL (Fig. 1) (R^2= 0.20; p<0.01). Vagile benthic fauna was found in 36% of individuals and was mainly present in individuals from 25 to 50 mm SL (Fig. 1). However its average contribution to the stomach content was lower than zooplankton. Undetermined items were found in 96% of individuals, and may represent a non-negligible part of stomach contents (Table 1).

Stable isotopes composition of food sources and their potential consumers

No significant difference was found in $\delta^{15}N$ values of potential food sources (Kruskal-Wallis test, p >0.05) (Fig. 2). On the other hand, food sources varied significantly in their $\delta^{13}C$ values (Kruskal-Wallis test, p <0.05). There were significant differences between $\delta^{13}C$ values of *Sargassum* spp. and of the two dominant seagrasses *H. stipulacea* and *S. isoetifolium* (Dunn's Multiple Comparison Test, p<0.05; Fig. 2). $\delta^{13}C$ values of seagrasses differed significantly from those of zooplankton

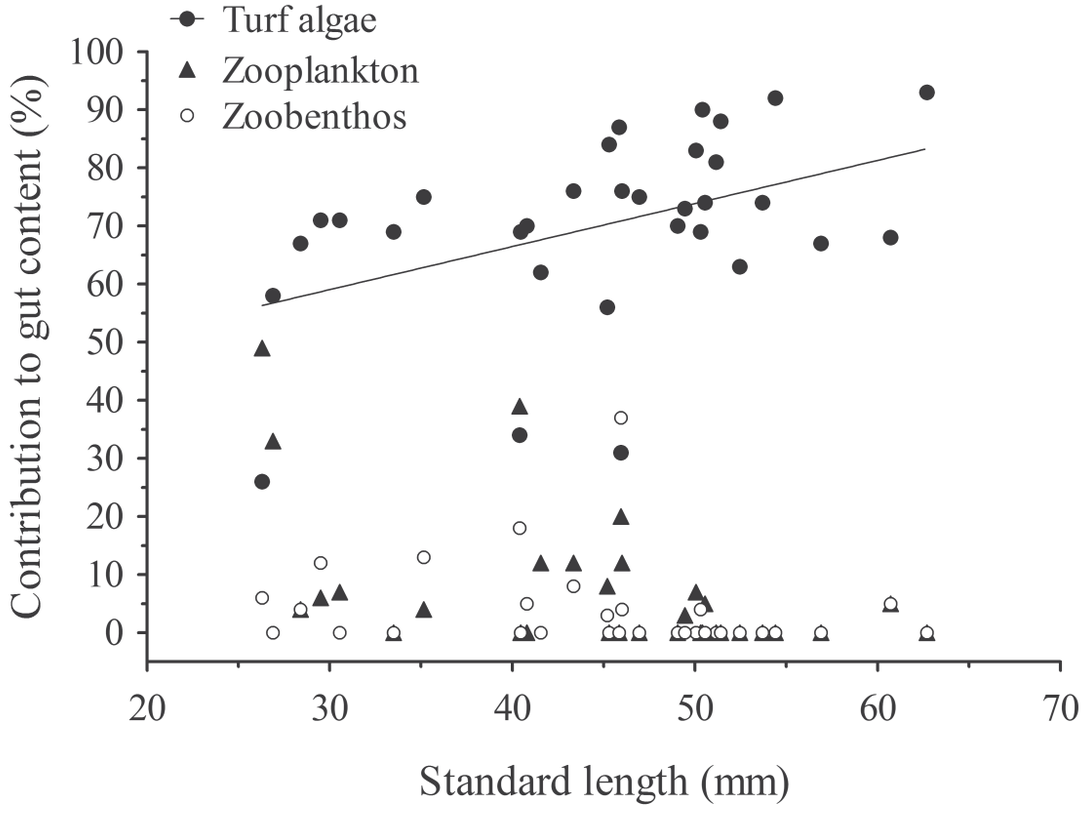


Fig. 1. – Contribution from three food categories (%) *vs* standard length (mm) of the footballer demoiselle, *Chrysiptera annulata* (n=33).

TABLE 1

Stomach content analysis (%) and occurrence frequency (%) in stomachs of *Chrysiptera annulata* (n=35).

	Turf algae	Zooplankton	Zoobenthos	Seagrass	Fish scales	Undetermined
Mean (± SD)	70.1±16	7.1±12.1	3.6±7.5	0.1±0.6	0.8±2.4	18.2±9.6
Minimum	25.7	0.0	0.0	0.0	0.0	0.0
Maximum	93.3	48.6	37.1	25	11.1	33.3
Occurrence frequency	97	48	36	6	12	94

(Dunn's Multiple Comparison Test, $p < 0.05$) but not from those of zoobenthos. Zoobenthos $\delta^{13}C$ values differed significantly from those of *Sargassum* spp. (Dunn's Multiple Comparison Test, $p < 0.05$). $\delta^{13}C$ values of associated organisms (i.e. seagrass epiphytes, *Sargassum* epiphytes or macroalgae growing within the territories) did not differ from each other or from their host plant, except *Padina* sp. whose $\delta^{13}C$ values differed significantly from those of *Sargassum* spp. and zooplankton (Dunn's Multiple Comparison Test, $p < 0.01$).

$\delta^{13}C$ and $\delta^{15}N$ values of *C. annulata* varied between -13.9 and -16.2 ‰ (mean ± S.D., -15.1 ± 0.6‰) and between 7.8 and 8.8 ‰ (mean ± S.D., 8.2 ± 0.2‰), respectively (Fig. 2). SIAR modelling output (Fig. 3) indicated that zoobenthos contributed the least in the diet of *C. annulata* (mean: 22%, 95% credibility interval 15-29%). Algae contribution to the diet was the more variable (mean: 35%, 95% credibility interval 23-48%) and zooplankton, upon average, represented the most important food sources in the *C. annulata* diet (mean: 43%, 95% credibility interval: 35-50%).

$\delta^{15}N$ values of *P. trilineatus* varied significantly between the two sampling locations (Mann-Whitney U test, $p < 0.05$; Fig. 2).

Isotopic niche parameters

There was relatively little overlap among the isotopic niches of the four damselfish species

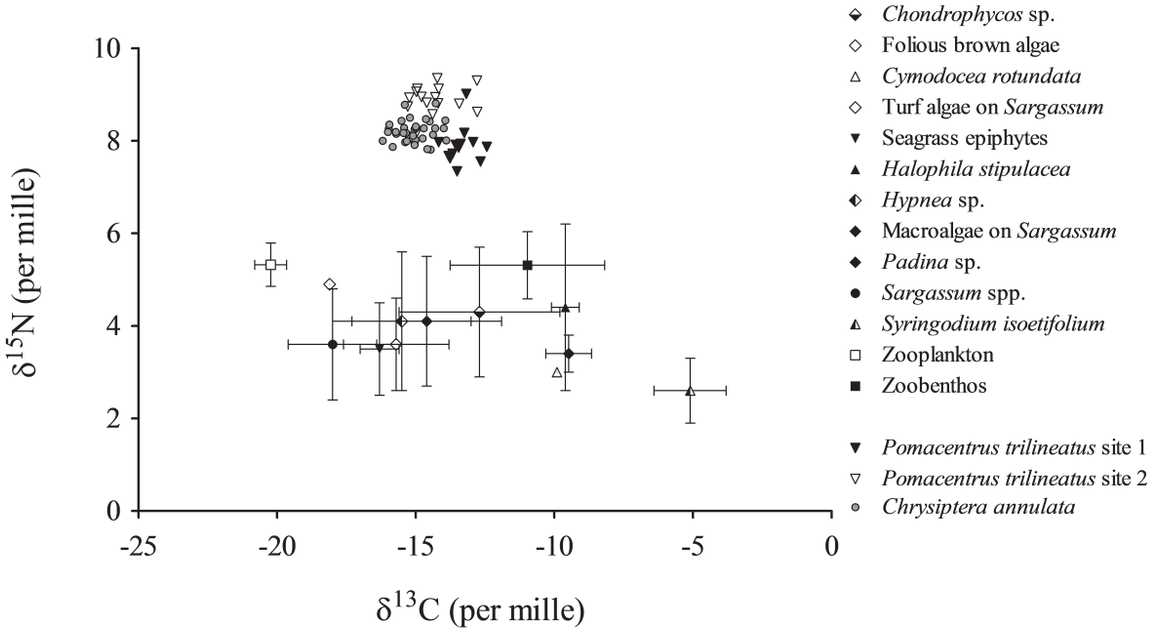


Fig. 2. – $\delta^{13}C$ vs $\delta^{15}N$ of potential food sources (mean ± sd), *Chrysiptera annulata* and the two populations of *Pomacentrus trilineatus* (individual data points, n= 35 and n=29, respectively).

(Fig. 4a). There was no overlap at all between the two groups of *P. trilineatus* or between *Pl. lacrymatus* and the four other groups. The highest overlap was between *C. annulata* and *C. unimaculata* (0.171 ‰^{2}, i.e. about 40% of the isotopic niches of both species). Standard ellipse area (SEA_b) of *C. annulata* was similar to the one of *C. unimaculata, Pl. lacrymatus* and the *P. trilineatus* of location 1, but was smaller than the one of the *P. trilineatus* of sampling location 2 in 97.44% of model estimates (Fig. 4b).

DISCUSSION

Analyses of stomach contents and stable isotopes demonstrate that *C. annulata* is an omnivorous/herbivorous species heavily relying on algal resources on the Great Reef of Toliara. Our findings on the animal contributions to stomach contents are in agreement with previous studies (Harmelin-Vivien, 1979; Allen & Randall, 1980) but show also that turf algae are the dominant food item ingested by all individuals, except by the smallest ones. This is not astonishing since turf algae are often dominant in the stomach contents of omnivourous/herbivorous damselfishes (Jones et al., 2006; Frédérich et al., 2009; Dromard et al., 2013). On the other hand, stable isotopes and SIAR modelling suggested that planktonic, and to a lesser extent, benthic animals were also important contributors to the diet of the footballer demoiselle. Three non-exclusive hypotheses can explain these apparent discrepancies. Firstly, stomach contents indicated that small individuals ingest more animal preys than do larger ones, and that turf algae progressively dominated the diet during growth. Measured isotopic compositions in fish muscle represent integration from the fish diet probably over a period of 1-2 months, as opposed to stomach contents, which are snapshots of the diet (e.g. Caut et al., 2010). Therefore, even for large individuals, stable isotope composition could reflect the contribution of animal consumption from past month(s). Secondly, stable isotopes

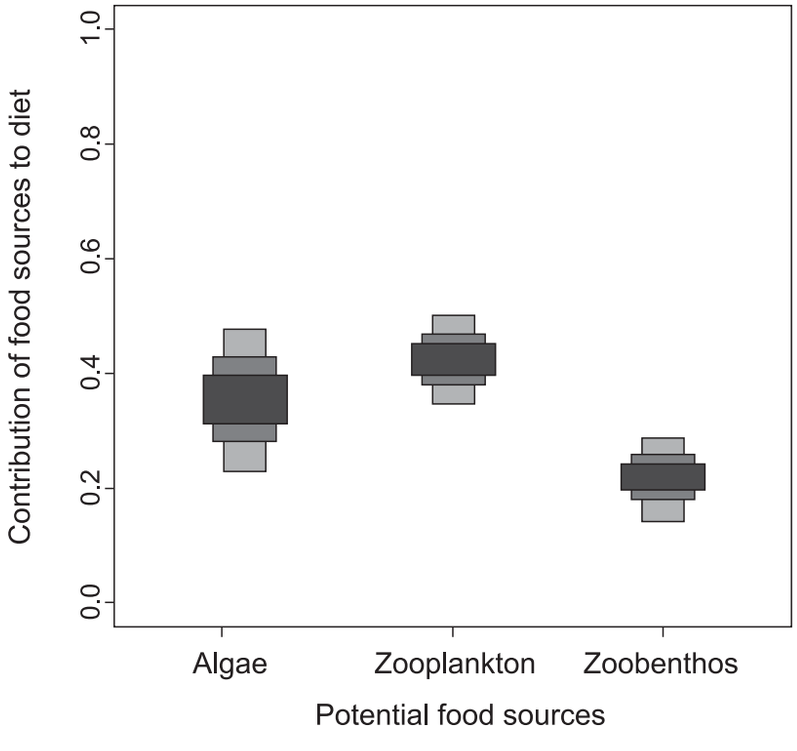
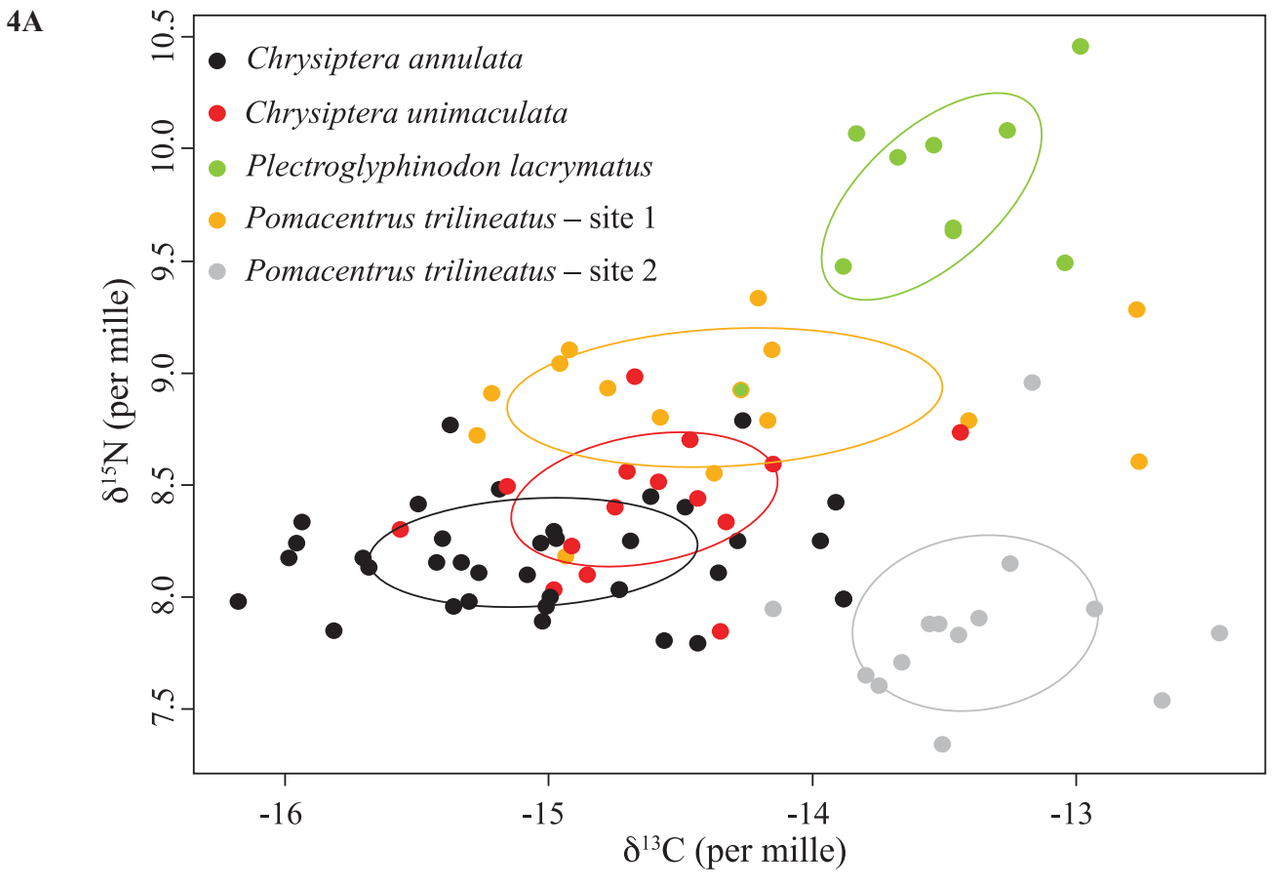


Fig. 3. – Boxplots showing relative contributions of potential food items to *Chrisyptera annulata* diet, computed using the SIAR model. Dark, median and light grey boxes are respectively the 50%, 75% and 95% credibility intervals of the probability of density function distributions of the model solutions



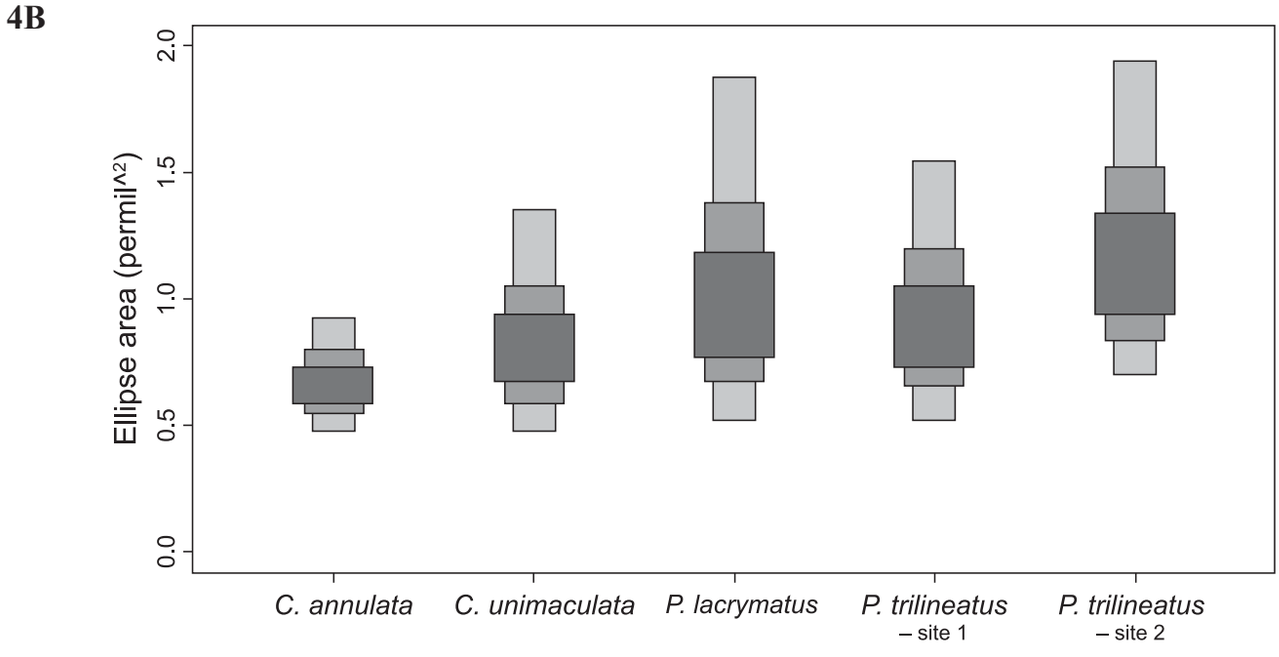


Fig. 4. – (a) Isotopic niches of *Chrysiptera annulata*, *Chrysiptera unimaculata, Plectroglyphidodon lacrymatus* and *Pomacentrus trilineatus* groups 1 and 2. Solid lines represent the bivariate standard ellipses associated to each group. (b) Boxplots of model-estimated bivariate standard ellipse area (SEA_b). Dark, median and light grey boxes are respectively the 50%, 75% and 95% credibility intervals of the probability of density function distributions of the model solutions, and black dots are the modes of these distributions.

focus on the assimilated part of the diet rather than the ingested one. The animal fraction could be more easily digested than algae and therefore could be rapidly degraded (KRAMER et al., 2013), contributing to the undetermined part of the stomach content (between 0 and 33% of examined stomach contents). Thirdly, these undetermined items could be processed animal tissues but also detritus of animal origin (e.g. faeces) or mucus produced by corals embedded with organic debris (WILSON et al., 2003). Such material is known to compose sometimes a large part of the diet in coral reef fishes (WILSON et al., 2003). Here, these sources were impossible to take into account in our SIAR modelling, but DROMARD et al. (2013) reported contributions of about 30% of detritus in the diet of *Stegastes planifrons* and *Stegastes adustus*.

The diet of our species may be qualified as omnivorous with an important contribution of algal material. Such mix between animal and vegetal food sources is not uncommon in damselfishes (FRÉDÉRICH et al., 2009). In farming species such as *Stegastes nigricans*, HATA & UMEZAWA (2011) observed a higher abundance of animal prey inside damselfish territory than outside, suggesting that these animals were also gardened and contributed significantly to *S. nigricans* diet. Other herbivorous fishes such as Scaridae also massively ingest the animals (mainly crustaceans) associated with turf algae (KRAMER et al., 2013). This supplementary food may provide adequate nutritional balance to individuals, with algae providing energy and animals supplying proteins (RAUBENHEIMER et al., 2005). The animal contribution to the diet of *C. annulata* decreases during growth and thus, the balance of animal and vegetal food sources varies across its ontogeny. Such ontogenetic diet variation has also been highlighted in other damselfish species (FRÉDÉRICH et al., 2010).

We also questioned if consumption of seagrass could represent an unexpected specialization of the footballer damselfish. Seagrasses in stomach contents were infrequent and represented only small amounts of material, suggesting "accidental" ingestion. Seagrasses were not a target food source for *C. annulata*, as stomachs of specialized seagrass grazers are generally full of seagrass pieces (HAVELANGE et al., 1997; GULLSTRÖM et al., 2011). Therefore, *C. annulata* did not use seagrasses as a food source but used meadows mainly as habitat. More importantly than seagrasses, the presence of small coral or rock boulders is determinant for the habitat of *C. annulata*, allowing the delimitation of small territories for it to defend. This fits the habitat description given by FISHELSON (1998) and the one observed in Mayotte (WICKEL et al., 2014) and Tanzania (GARPE & ÖHMAN, 2003). It might also explain why *C. annulata* may be found elsewhere on the reef flat than in seagrass meadows. In summary, inhabiting seagrass meadow is not linked to a trophic specialisation of this territorial species but is more likely a habitat divergence compared to other territorial herbivorous damselfishes.

Few genera of damselfish are territorial and practice gardening (HATA & KATO, 2002; CECCARELLI et al., 2005; JONES et al., 2006) but this peculiar farming behaviour evolved several times during the evolutionary history of Pomacentridae (FRÉDÉRICH et al., 2013). Considering its territoriality, it is legitimate to examine if *C. annulata* may be considered as a gardener. In *Stegastes* spp., this gardening may ultimately lead to a *Polysiphonia* sp. monoculture (HATA & KATO, 2004). Although we did not detail the algal species present on each territory, it is evident that it was not a monoculture as in some *Stegates* spp. However, the fact that each *C. annulata* conserved large algae without consuming it on each territory could be evidence of another type of gardening behaviour where macroalgae are conserved to allow gardening of their epiphytes and where algal community diversity, abundance and successional pattern are significantly affected by the fish (CECCARELLI et al., 2005). In this intermediary case, gardening does not necessarily result in a monoculture as in *Stegastes nigricans* (HATA & KATO, 2002).

Our second objective was to compare the isotopic niches of four damselfishes displaying

an important contribution from algae in their diet. There was little overlap among the isotopic niches of the four studied species, except between the two *Chrysiptera* species. The weak overlap among species could indicate that they differed in terms of trophic resource use, although algae constitute a large part of their food (FRÉDÉRICH et al., 2009). Differences in these isotopic niches may be related to trophic differences (i.e. different algal species, different proportion of animals in the diet) but also to trophic behaviour (i.e. farmers vs. foragers) and habitats (coral boulders, seagrass meadows, "vasques"). Indeed, isotopic niche variability is also determined by isotopic variability of sources and this one could be related to spatial variability (FLAHERTY & BEN-DAVID, 2010). This was documented in the present study by the two populations of *P. trilineatus* differing significantly in their isotopic niches, probably partly because the isotopic composition of their food sources differs spatially. Nevertheless, we cannot reject the possibility that the two populations of *P. trilineatus* also differed in their trophic exploitation. In summary, isotopic niche must be seen as an integration of two axes of ecological niche: the trophic and the habitat axis (NEWSOME et al., 2007; FLAHERTY & BEN-DAVID, 2010).

Despite very different habitats, the isotopic niches of the two *Chrysiptera* species overlapped significantly, showing they shared more food resources with each other than with *P. trilineatus* and *P. lacrymatus*. The two *Chrysiptera* species have a similar behaviour (i.e. defence of a small territory with algal cover) and, therefore, habitat difference in the Toliara great reef (seagrass meadow vs. coral rubbles) between these two species could be essential to mitigate interspecific competition. Such potential competition among species is also observed for gardeners of *Stegastes* genus (DROMARD et al., 2013). For the footballer demoiselle being one of the smallest damselfish in the area, inhabiting seagrass meadows could be a way to escape competition with other territorial damselfishes.

CONCLUSIONS

Chrysiptera annulata is an omnivorous species, mostly relying on algal material but complementing its diet with zooplankton and small zoobenthic organisms. Seagrass does not contribute significantly to its diet. Nevertheless, inhabiting seagrass meadow of the reef flat may be a way to reduce competition with other territorial herbivorous damselfishes that display similar trophic strategies. Our study revealed ecological variations among territorial damselfishes and efforts to study their role in this ecosystem must be encouraged.

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Change in Historical Range of the Ural Owl in Europe

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ABSTRACT. A carpometacarpus recovered during archaeological excavations in the town of Quaregnon is the westernmost find ever reported in Europe of a Ural Owl (*Strix uralensis*), and the first occurrence for Belgium. Both the morphology of the skeletal element and its measurements rule out an identification as any of the other Strigiformes from the Western Palearctic. The provenance of this specimen, that dates to the medieval period (10th-12th centuries AD), is discussed. It is hypothesized that the bird was a wild animal, but the available evidence does not unequivocally determine whether it belonged to a local, breeding population that went extinct or if it came from a more distant population. However, a survey of other zooarchaeological finds of Ural Owl in Europe shows that the species occurred farther west in the past, outside the present natural breeding range. This suggests that Ural Owl may have found suitable nesting biotopes in Belgium and northern France during the medieval period.

KEY-WORDS: biogeography, bird, *Strix uralensis*, Ural Owl, zooarchaeology

INTRODUCTION

Faunal remains recovered from archaeological excavations allow reconstruction of subsistence strategies of past human populations, but also provide information on the ancient distribution of animal species. In this respect, when studying archaeological bird remains, it is important to keep in mind that species may occur that are not part of the current local avifauna (Stewart, 2005). Zooarchaeological evidence for taxa that are not native to the region of the archaeological site where they were discovered can often be explained as a result of trade. Alternatively, zooarcheological findings can indicate changes in species distribution ranges through time, either due to climatic or anthropic factors. For example, Nikulina & Schmölcke (2015) used subfossil bones to show that birds of the genus *Pelecanus* occurred far out of their present range between 7.4 and 5.0 ka BP (thousand years before present) in the Danish archipelago. This type of information is valuable for the documentation of climate change and human impacts through time, and can be relevant to conservation biology (Lyman, 2006; for a recent example see Stewart, 2007). Here we collate archaeological finds of a strigiform species and discuss its zoogeographical relevance after the compilation of zooarchaeological reports from Europe that are not easily accessible to the wider ornithological community.

In 2008 and 2009, archaeological excavations were carried out by the Service public de Wallonie at the 'Grand' Place' of Quaregnon (Denis, 2010; Denis, 2011), a town situated in the southwest of Belgium at ~6 km west of the city of Mons (Fig. 1). Quaregnon (50°26'34" N, 3°51'56" E) is located in the Haine valley, at 33 meters above sea level. During the 2008 campaign, a carpometacarpus of a strigiform was discovered in a medieval settlement. The ditch surrounding an ancient church, in which

this bone was found also yielded a few remains of the usual food animals (cattle, pig, sheep, chicken) and some human bones from nearby disturbed graves (GOFFETTE et al., 2015). Unlike the other animal remains, which are considered as human consumption refuse, the strigiform bird is believed to be part of a discarded carcass (GAUTIER, 1987). Associated artefacts date the filling to the 9th-11th centuries AD.

MATERIAL AND METHODS

The bone discovered in Quaregnon was initially identified to genus level with the aid of the diagnostic criteria described in LANGER (1980) and by comparison with the modern reference collections of the Royal Belgian Institute of Natural Sciences (RBINS) and of the Royal Museum of Central Africa (RMCA), presently also housed at the RBINS. In addition to the measurements taken on the RBINS/RMCA specimens, other carpometacarpus dimensions were obtained from the Regalia collection (Institut de Paléontologie Humaine, Muséum national d'Histoire naturelle (MNHN), Paris) and from the Staatssammlung für Anthropologie und Paläoanatomie München (SAPM) for Ural Owl *Strix uralensis* PALLAS, 1771 and from the Institute of Systematics and Evolution of Animals (Polish Academy of Sciences (PAS), Warsaw) for Ural Owl and Short-eared Owl *Asio flammeus* (PONTOPPIDAN, 1763). Measurements were taken with digital callipers following the recommendations of VON DEN DRIESCH (1976). The descriptions of anatomical features below follow the nomenclature of BAUMEL (1993).

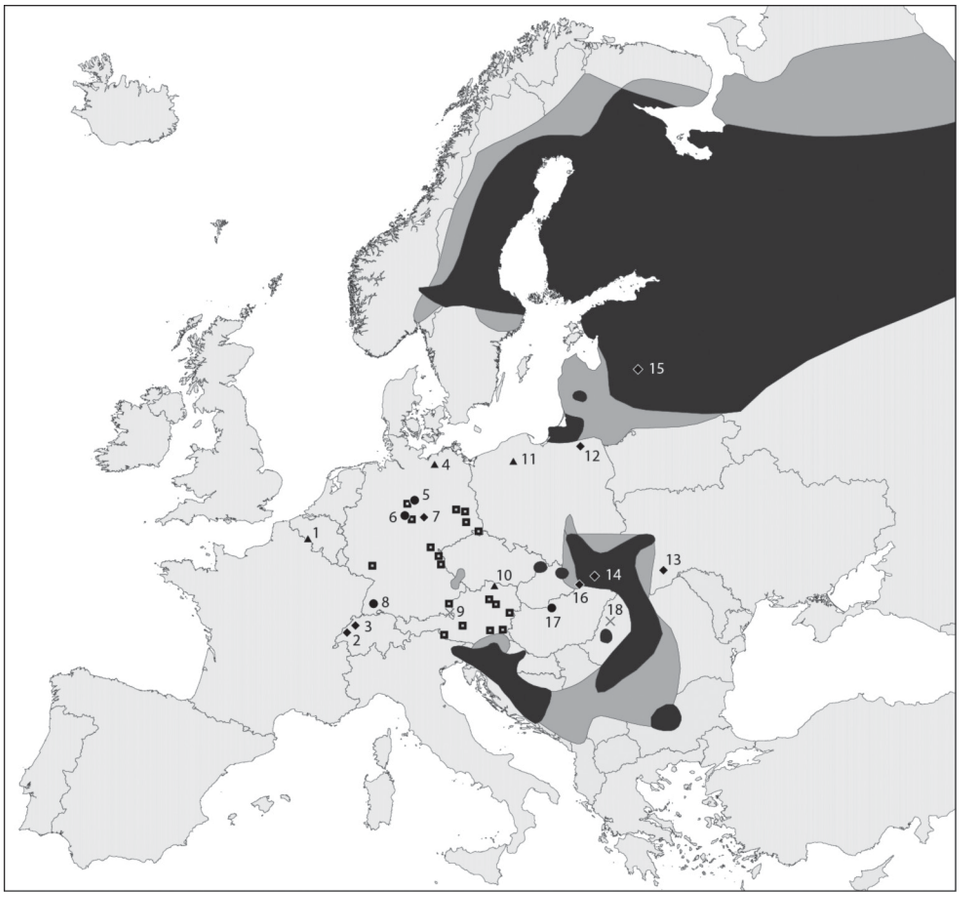

Fig. 1. – Present distribution of Ural Owl (black areas - main distribution range, grey areas - periphery of the distribution, hollow squares - single observations) and zooarchaeological discoveries in Europe (diamonds - 9000 BC-1000 BC, circle - 1000 BC-600 AD, triangle - 600-1800 AD, cross - indeterminate). The numbers correspond to those of the archaeological sites mentioned in Table 2. Map modified after SCHERZINGER (2006).

The strigiform bone has been radiocarbon dated in order to confirm its contemporaneity with the associated artefacts. Only the mid-part of the carpometacarpus was used, the two extremities are still intact and stored in the collections of the RBINS. No palaeogenetic analysis has been attempted on this bone thus far. It is believed that such molecular data would be of little help in determining its provenance because of the high genetic diversity observed within each of the present-day European populations of Ural Owl (HAUSKNECHT et al., 2014).

RESULTS

The strigiform bone discovered is an almost complete left carpometacarpus of which only the proximodorsal part of the *os metacarpale minus* was slightly damaged (Fig. 2). The fresh aspect of the fractured part suggests that it was broken during the excavation. The bone surface is very well preserved, but no anthropogenic traces (e.g., cut marks) were observed despite careful examination with binocular microscope (magnification 20 x). When comparing the specimen with the skeletons of strigiforms presently found in Belgium, it appears that the Short-eared Owl is the closest in size. However, the carpometacarpus from Quaregnon appears less elongated and more robust because of the shorter and wider *spatium intermetacarpale*. The *os metacarpale majus* is also wider and less straight, because the lateral border slightly curves laterally close to the *processus alularis*. The *os metacarpale minus* is thicker and curved. The distal epiphysis is larger and more robust compared to that of the Short-eared Owl. In particular the *facies articularis digitalis major* is more robust and is laterally more protruding. The *facies articularis digitalis minor* projects more in a distal direction compared to the Short-eared Owl. In proximal view, the *trochlea carpalis* is thick and the two cristae of the trochlea are almost parallel, unlike in Short-eared Owl where the dorsal crista is oblique in a laterodorsal direction. The aforementioned morphological features of the bone are typical of the genus *Strix*.

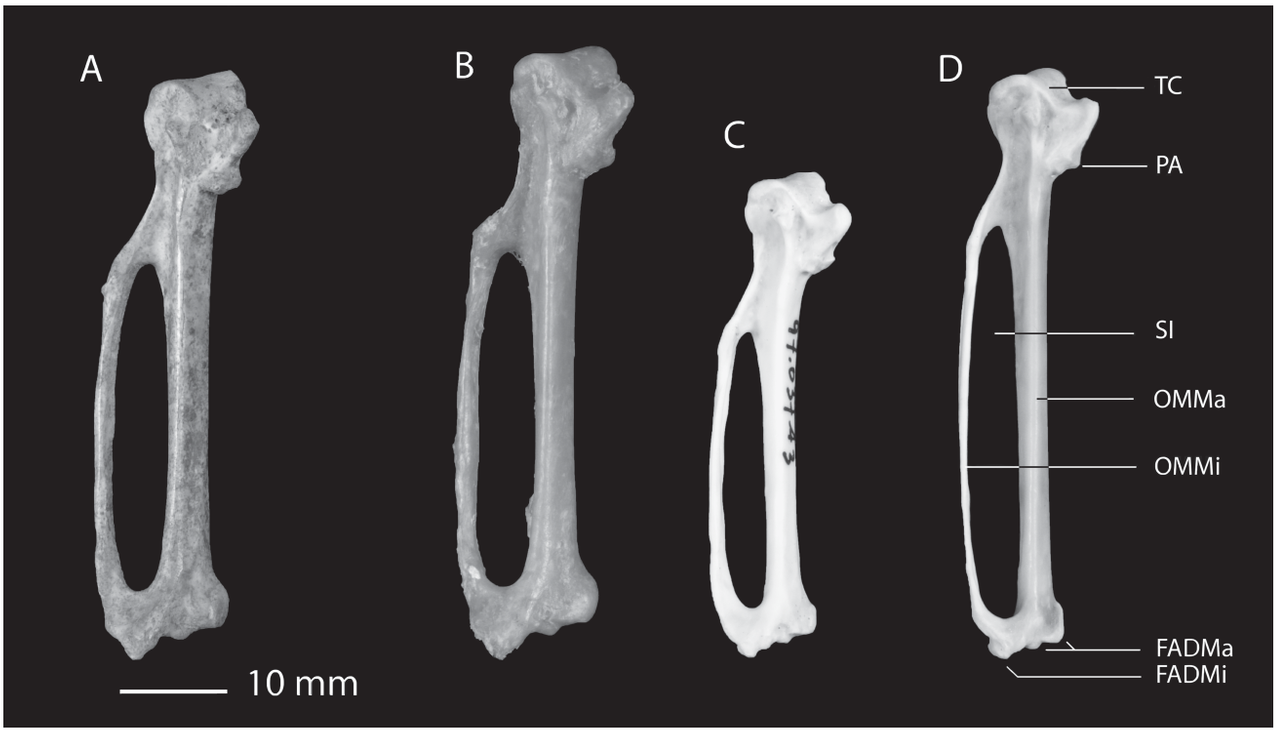

Fig. 2. – Carpometacarpus from: **A.** Ural Owl from Quaregnon. **B.** Modern Ural Owl (RBINS 20655). **C.** Modern Tawny Owl (RMCA 97037A3). **D.** Modern Short-eared Owl (RBINS 80531). TC - *Trochlea carpalis*, PA - *Processus alularis*, SI - *Spatium intermetacarpale*, OMMa - *Os metacarpale majus*, OMMi - *Os metacarpale minus*, FADMa - *Facies articularis digitalis major*, FADMi - *Facies articularis digitalis minor*.

Within the genus *Strix*, four species occur nowadays in the Western Palearctic (*sensu* CRAMP & SIMMONS, 1977): Tawny Owl *Strix aluco* L., 1758, Ural Owl, Hume's Owl *Strix butleri* (HUME, 1878) and Great Grey Owl *Strix nebulosa* FORSTER, 1772. Comparisons with the reference collections of the RBINS/RMCA and with published measurements (LANGER, 1980) show that the carpometacarpus of the Tawny Owl is too small. Consequently, Hume's Owl, the smallest species of this genus in the Western Palearctic, can also be excluded. The Great Grey Owl, which is the largest *Strix* species in the Western Palearctic, is definitely too large compared to the Quaregnon specimen. However, the measurements of the archaeological carpometacarpus fall exactly within the variation of Ural Owl (Fig. 3 and Table 1). The metrical data also show that the Short-eared Owl, which was already excluded on a morphological basis, is somewhat smaller compared to the Quaregnon specimen and Ural Owl in general. To conclude, both morphological features and dimensions indicate that the fossil carpometacarpus from Quaregnon belongs to the Ural Owl.

The AMS radiocarbon date obtained directly on the Ural Owl bone from Quaregnon (993 ± 32 BP, RICH-21621) validates the relative dating based on the associated archaeological material. The calibrated results provide a chronological range between the 10th and 12th centuries AD, which is slightly more recent than the date indicated by the archaeological material (9th-11th centuries AD):

Calibrated (1 σ)	990 AD (60.7%) 1050 AD
	1090 AD (7.5%) 1120 AD
Calibrated (2 σ)	980 AD (95.4%) 1160 AD

DISCUSSION

The Ural Owl is a polytypic species that is nowadays widespread across the entire Palearctic (CRAMP, 1985; MEBS & SCHERZINGER, 2006). Within the Western Palearctic (Fig. 1) its range extends from Finland in the north to Bulgaria in the south and from Norway in the west to the eastern border of the Western Palearctic in the east (and further up to the Pacific Ocean, see DEL HOYO et al., 1999). Between 1976 and 1993, birds have been successfully reintroduced in Bavaria,

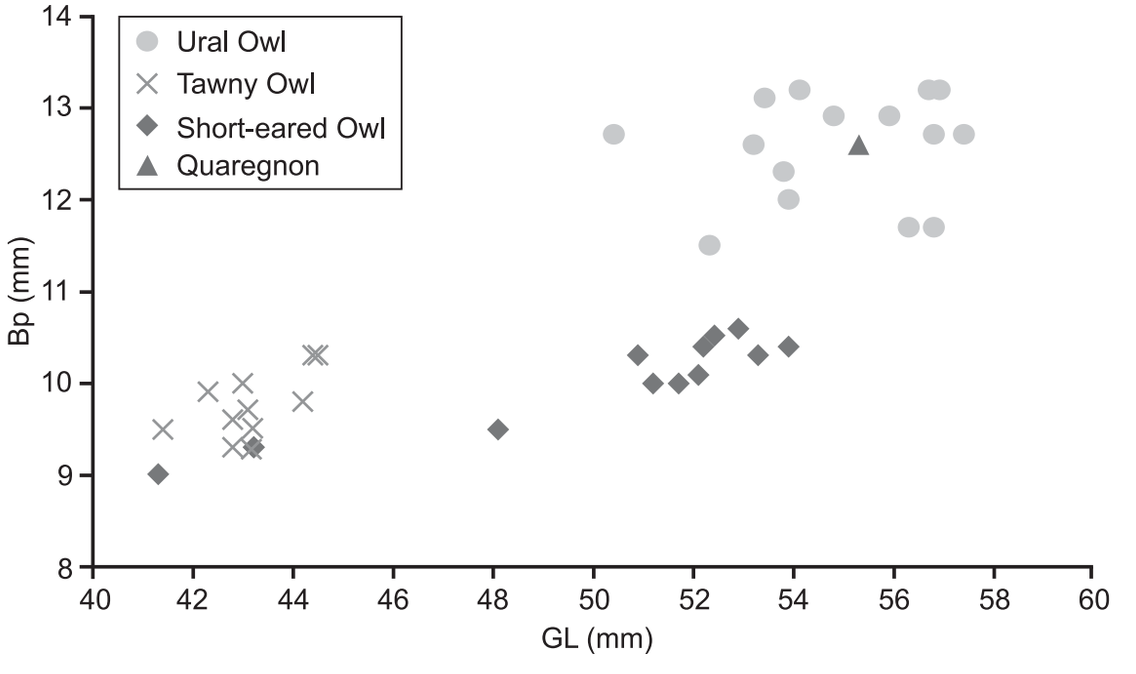

Fig. 3. – Plot of the greatest breadth of the proximal extremity (Bp) and the greatest length (GL) of the Ural Owl carpometacarpus from Quaregnon and those of modern specimens of Ural Owl, Tawny Owl and Short-eared Owl (list of specimens and measurements in Table 2).

TABLE 1

Measurements (in mm) of the Ural Owl carpometacarpus from Quaregnon, compared to those of museum specimens of the same species and of Short-eared Owl, Tawny Owl and Great Grey Owl. Measuring distances and their abbreviations are according to VON DEN DRIESCH (1976): GL = Greatest length; Bp = Greatest breadth of the proximal extremity; Did = Diagonal of the distal end. MNHN = Regalia collection, Institut de Paléontologie Humaine, Muséum national d'Histoire naturelle, Paris; PAS = Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Warsaw; RBINS = Royal Belgian Institute of Natural Sciences, Brussels/Royal Museum for Central Africa, Tervuren; SAPM = Staatssammlung für Anthropologie und Paläoanatomie, München. Measurements ranges for Great Grey Owl are from CAMPBELL & BOCHEŃSKI (2010).

Species	Specimen n°	GL	Bp	Did
Ural Owl (*Strix uralensis*)	Quaregnon Z01 F97 SU01.242	55.3	12.6	10.8
Ural Owl (*Strix uralensis*)	PAS 5752/99	56.6	12.3	11.8
Ural owl (*Strix uralensis*)	PAS 2767/73	53.8	11.7	10
Ural owl (*Strix uralensis*)	PAS 7290/10	56.8	12.7	11.5
Ural owl (*Strix uralensis*)	PAS 6787/07	57.4	12.9	11.1
Ural owl (*Strix uralensis*)	PAS 6000/02	55.9	13.2	11.8
Ural owl (*Strix uralensis*)	PAS 6786/07	56.7	12.7	11.5
Ural owl (*Strix uralensis*)	MNHN 569	56.8	13.2	11.4
Ural owl (*Strix uralensis*)	MNHN 689	56.9	13.2	11.3
Ural owl (*Strix uralensis*)	MNHN 970	54.1	11.7	10
Ural owl (*Strix uralensis*)	RBINS 20655	56.3	12.9	11.4
Ural owl (*Strix uralensis*)	SAPM 6	54.8	13.1	11.2
Ural owl (*Strix uralensis*)	SAPM 7	53.4	12	10.9
Ural owl (*Strix uralensis*)	SAPM 8	53.9	12.7	10.9
Ural owl (*Strix uralensis*)	SAPM 9	50.4	11.5	10.3
Ural owl (*Strix uralensis*)	SAPM 10	52.3	12.6	10.9
Ural owl (*Strix uralensis*)	SAPM 11	53.2	13	11.2
Tawny Owl (*Strix aluco*)	RBINS 93030A01	43.2	9.5	8.5
Tawny Owl (*Strix aluco*)	RBINS 92017A03	43.2	9.3	8.5
Tawny Owl (*Strix aluco*)	RBINS 99062A03	43	10	8.6
Tawny Owl (*Strix aluco*)	RBINS 82574	44.4	10.3	9.2
Tawny Owl (*Strix aluco*)	RBINS 97048A19	44.2	9.8	8.5
Tawny Owl (*Strix aluco*)	RBINS 97037A03	44.5	10.3	8.7
Tawny Owl (*Strix aluco*)	RBINS 96004A03	43.1	9.7	8.4
Tawny Owl (*Strix aluco*)	RBINS 82611	44.2	9.8	8.8
Tawny Owl (*Strix aluco*)	RBINS 82716	42.8	9.3	8.3
Tawny Owl (*Strix aluco*)	RBINS 99086A06	42.3	9.9	8.4
Tawny Owl (*Strix aluco*)	RBINS 77242	41.4	9.5	8.2
Tawny Owl (*Strix aluco*)	RBINS 96009A01	42.8	9.6	8.6
Great Grey Owl (*Strix nebulosa*)	number of specimens = 8	60.1-68.2	13.6-15.6	10.9-12.6
Short-eared Owl (*Asio flammeus*)	PAS 5479/96	52.1	10.1	7.2
Short-eared Owl (*Asio flammeus*)	PAS 2533/72	51.2	10	6.9
Short-eared Owl (*Asio flammeus*)	PAS 3084/75	48.1	9.5	6.9
Short-eared Owl (*Asio flammeus*)	PAS 5745/99	43.2	9.3	7.3
Short-eared Owl (*Asio flammeus*)	PAS 3083/75	41.3	9	7.1
Short-eared Owl (*Asio flammeus*)	PAS 7085/08	51.7	10	7.6
Short-eared Owl (*Asio flammeus*)	RBINS 92138A04	52.2	10.4	7.4
Short-eared Owl (*Asio flammeus*)	RBINS 96074A07	52.9	10.6	7.4
Short-eared Owl (*Asio flammeus*)	RBINS 77291	52.4	10.5	7.8
Short-eared Owl (*Asio flammeus*)	RBINS 80531	53.9	10.4	7.9
Short-eared Owl (*Asio flammeus*)	RBINS 81084	50.9	10.3	7.4
Short-eared Owl (*Asio flammeus*)	RBINS 80532	53.3	10.3	7.8

Germany, where the species was breeding until 1925, and the same has been done in the Czech Republic from 1995 onward (MEBS & SCHERZINGER, 2006). Ural Owls are sometimes observed outside their present breeding range (Fig. 1), as was the case in western Germany or in north-eastern Italy where the species bred in 1994 (MEBS & SCHERZINGER, 2006).

The present, highly fragmented distribution in Western Europe is believed to represent the relics of a wider distribution during the last glacial period (MEBS & SCHERZINGER, 2006; BASHTA, 2009). In addition, the species' distribution was still contracting as late as the beginning of the 20th century AD, especially because of human persecution (SCHERZINGER, 2006). The striking patchiness of the current populations would thus represent the remaining suitable biotopes acting as cryptic southern refugia (STEWART et al., 2010) where the species has not been too much disturbed, including mainly semi-mountainous areas in western and southern Europe.

To explain the presence of the Ural Owl bone in Quaregnon, two possibilities need to be considered: a captive bird brought in by humans or a wild bird. Firstly, the specimen may have been brought in by humans from another area within the natural breeding range of the species. The find from Quaregnon dates to a period when human population movements and long distance trade were frequent in Europe. Those networks of transfers of goods and people included regions where the Ural Owl breeds today, such as Fennoscandia and the Baltic countries. People travelling from those areas to Western Europe could have been carrying either a living Ural Owl or a whole or partial carcass. In medieval times, birds of prey were sometimes traded and transported over long distances to be used for falconry (OGGINS, 2004). Evidence for such practices has been found, for instance, in Winchester (England) where bones of at least two Gyrfalcons *Falco rusticolus* L., 1758 were discovered in archaeological contexts dating to the 11-12th centuries AD. The birds are believed to have been imported from Norway or Iceland (SERJEANTSON, 2006). Although many species have been trained for falconry, we found no data in the literature referring to the use of Strigiformes for this purpose in Europe. Owls, probably mainly Eurasian Eagle Owl *Bubo bubo* (L., 1758) have been exploited as lures to hunt other bird species (JAQUES & DOBNEY, 2002; TYRBERG, 2002) but not to catch prey. It therefore seems unlikely that the Ural Owl from Quaregnon was brought in as a captive bird meant for falconry. Alternatively, it could have been imported as a curiosity or a pet, but no evidence for this is available. Moreover, it should be underlined that no artefacts were found on the entire archaeological site that could attest the inhabitants possessed items obtained through long distance trade.

The other possibility involves a bird of wild origin. Although the Ural Owl nests preferentially in coniferous forests within the northern and eastern part of its range and in beech (*Fagus* sp.) forests in its southern distribution area, it is relatively tolerant to the tree species composition of its habitat (CRAMP, 1985; MEBS & SCHERZINGER, 2006). It avoids intensively exploited forests where human disturbance is strong. Such forests are unlikely to provide suitable nesting places, such as broken tree stumps or hollow trunks where the nests are most frequently built (VREZEC & TOME, 2004). In this respect, the age of woodlands is of importance because trees need to be large and thus old enough to support the nests (LUNDBERG & WESTMAN, 1984; BOLBOACA et al., 2013). Altitude seems of less importance since nests have been recorded from 160 m.a.s.l. in Slovakia up to 1600 m.a.s.l. in Romania (CRAMP, 1985; KRISTIN et al., 2007). As the species requires open places to hunt, nests are generally situated near woodland margins or within clearances (MEBS & SCHERZINGER, 2006). CRAMP (1985) notes that this owl is commonly found near human settlements, and that it favours extensive cultivation and pasture land. It is sometimes found within towns, particularly during winter.

Thus far archaeobotanical data that could document the medieval or post-medieval

environment of Quaregnon are lacking, but general information on the historical land cover in the region suggests that the species may have found suitable habitats to nest. During the early Roman period, woodlands in the southern part of Belgium suffered significant degradation. Towards the end of the Roman period and at the beginning of the medieval period (Merovingian times) forests recovered but deforestation started again later in the Early Middle Ages (Carolingian period) (VANPOUCKE et al., 2007). However, historical sources indicate that vast deciduous forests still existed south of Quaregnon during the medieval period and until the 16th century AD (VERHULST, 1999). This type of old deciduous forests probably met the ecological requirements of the Ural Owl. Even if some clearance of woodland had taken place, this would not necessarily have been detrimental to the species as it takes advantage of the newly created open landscapes to prey on small mammals (MEBS & SCHERZINGER, 2006). Shortly after, intensive logging started and at the end of the 18th century, as shown by the maps of FERRARIS (1771-1778), the landscape was turned into agricultural land almost comparable to the present state.

Further supportive of a local origin of the Quaregnon specimens is that Ural Owls have been found to be extremely sedentary, very rarely wandering outside their breeding range (MIKKOLA, 1983). Indeed, Finnish ringing records comprising a total of 58410 Ural Owls collected between 1913 and 2012 show that more than 90% of Ural Owls breed within a radius of 3 km from year to year (i.e. in the same territory). In Finland, the longest distance between two successive nest sites has been ~300 km (VALKAMA et al., 2014). However, some Ural Owls have been reported to occasionally travel up to more than a thousand kilometres (DORNBUSCH, 1990) and in Siberia, such long distance movements are not uncommon (MIKKOLA, 1983; MEBS & SCHERZINGER, 2006; CRAMP 1985). Therefore, although unlikely, it cannot be totally excluded that the bird discovered in Belgium could have been a migratory or vagrant bird.

Other zooarchaeological finds are known of Ural Owl outside its present-day breeding range. We compiled the Holocene finds listed in larger inventories such as those of PIEHLER (1976), of KESSLER (2014) and of von den DRIESCH & PÖLLATH (2010, see also BENECKE, 1999) with other data we found in the zooarchaeological literature. Find localities are indicated on Fig. 1 and detailed in Table 2, and these data support the suggestion made by several authors that the breeding range of the Ural Owl was more extensive in the past, particularly to the west (VOOUS, 1962; SAUER-NEUBERT, 1968; BECKER & PIEPER 1982; PUCHER & SCHMITZBERGER, 1999). The species was likely present in Switzerland at least during the 4th millennium BC, and was probably widespread in Germany during the first millennium AD and possibly even during the medieval period.

CONCLUSION

The hypothesis that the carpometacarpus from Quaregnon represents a native bird is considered very probable, either as a migratory/vagrant individual or as a local breeder. This is reinforced by other zooarchaeological finds outside the current breeding range in places broadly comparable to the Belgian locality in terms of latitude, altitude and habitat type. All these finds of Ural Owl support a wider westward distribution in the past, which was in all likelihood less fragmented than today and may have been continuous up to Western Europe. More zooarchaeological finds are necessary to further strengthen this hypothesis, and should ideally include bones of young birds, which would provide evidence that the Ural owl was breeding in the vicinity of the site.

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TABLE 2

Ural Owl remains discovered in Europe. The precision concerning the dates and preserved elements is variable and depends on the information found in the literature (C^{14} - radiocarbon dating).

No.	Country	Site	Age	Dates	Source	Skeletal element
1	Belgium	Quaregnon (Grand'Place)	Early-High Medieval Period	993 ± 32 BP (C^{14})	Goffette et al., 2015	1 carpometacarpus
2	Switzerland	Concise	Middle Neolithic	3692-3675 BC	Chiquet, 2009	1 femur
3	Switzerland	Twann	Neolithic	~3800 BC	Becker, 1981; Becker & Pieper 1982	1 carpometacarpus
4	Germany	Groß Raden	Medieval Period	9th-10th century AD	Gehl, 1981	1 humerus
5	Germany	Gielde (Am Hetelberg)	Roman Iron Age (- Early Medieval Period)	0-400 AD (-750 AD)	Schaal, 1968	1 tarsometatarsus
6	Germany	Göttingen (Abris Stendel XVIII)	Late Bronze Age - Iron Age	1000-500 BC	von den Driesch, 1994	1 element
7	Germany	Kyffhäuser (Höhle 2)	Late Bronze Age – Medieval Period	1200-900 BC or 500-1500 AD	Teichert & Lepiksaar, 1977	11 elements
7	Germany	Kyffhäuser (Höhle 7)	Bronze Age or Medieval Period	1800-900 BC or 500-1500 AD	Teichert & Lepiksaar, 1977	1 element
7	Germany	Kyffhäuser (Höhle 9)	Late Bronze Age	1200-900 BC	Teichert & Lepiksaar, 1977	1 element
8	Germany	Hüfingen	Roman Period	1st-2nd century AD	Sauer-Neubert, 1968	1 femur, 1 tibiotarsus
9	Germany	Karlstein	Bronze Age - Roman Period	not available	von den Driesch, 1979	2 ulna (same young bird)
10	Austria	Raabs an der Thaya	Medieval Period	10th century AD	Pucher & Schmitzberger, 1999	1 carpometacarpus, 1 femur
11	Poland	Człuchów	Late Medieval Period - Early modern period	second half 14th – end 17th century AD	Nogalski & Salaciak, 1990	1 humerus
12	Poland	Dudka	Early Mesolithic	9000-8500 BP	Tomek & Gumiński, 2003	1 element
12	Poland	Dudka	Late Mesolithic	7000-6000 BP	Tomek & Gumiński, 2003	1 element
12	Poland	Dudka	Neolithic	4700-4200 BP	Tomek & Gumiński, 2003	1 element
13	Ukraine	Polivanov Jar	Neolithic	4000-3500 BC	Voinstvenskij, 1967	1 element
14	Slovakia	Košice (Barca)	Bronze Age	1800-1300 BC	Ambros, 1992	2 elements
15	Latvia	Zvidze	Early Neolithic	site: between 6535±60 and 5320±50 (C^{14})	Strazdinja, 1986; Loze, 1988	not available
16	Hungary	Aggtelek	Neolithic	6000-3000 BC	Jánossy, 1977	not available
17	Hungary	Pilismarót-Malompatak	Roman Period	370-410 AD	Jánossy, 1985	1 tarsometatarsus
18	Romania	Vadu Crișului (Peștera suspendata)	Holocene	not available	Kessler, 1985	not available

Region. We thank Cécile Mourer-Chauviré (French National Centre for Scientific Research) and Zbigniew Bocheński (Polish Academy of Sciences) for their feedback on the bone find and for providing us with measurements of modern bones stored respectively in the Regalia collection of the Institut de Paléontologie Humaine, Muséum national d'Histoire naturelle in Paris and in the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences in Warsaw. We also thank Baudouin Van den Abeele (UCLouvain) for informing us about the medieval trade in birds of prey as well as about their use for falconry, and Norbert Benecke (Deutsches Archäologisches Institut) and Jeno Kessler (Eötvös Lorand University Budapest) for giving us complementary information on some finds. Finally, we would like to thank Anne-Marie Wittek (ADIA, Association pour la Diffusion de l'Information Archéologique) for modifying the map in Figure 1, as well as John R. Stewart (Bournemouth University), Didier Vangeluwe (RBINS) and Alain de Broyer (Aves/Natagora) for their valuable comments on a previous version of the manuscript and the reviewers for their constructive suggestions.

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Genetic monitoring of the endangered Pyrenean desman (*Galemys pyrenaicus*) in the Aude River, France

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ABSTRACT. The Pyrenean desman (*Galemys pyrenaicus*) is a small semi-aquatic mammal endemic to the Pyrenean Mountains and the northern half of the Iberian Peninsula. This species is currently considered as vulnerable in the IUCN Red List and has been suffering from habitat loss and fragmentation for decades but little is known about the impact of water flow modifications induced by hydroelectric power plants. In order to address this issue we monitored Pyrenean desman individuals living in a harnessed section of the Aude River, by genotyping both faeces samples and hair of live-trapped animals. During a three-year study (2011-2013), a total of 39 individuals were identified using 24 microsatellite loci, 28 from faeces and 11 from trapped animals. Several long distance movements were evidenced up to at least 15 km, a distance that has never previously been reported. These movements might be related to modifications of the river bed caused by very high water flows that occurred during the repair of the Nentilla hydroelectric plant. The local population density suggests that the Aude River provides suitable habitat for the Pyrenean desman, and preservation of this habitat should be a priority for the conservation of this species.

KEY WORDS: conservation, *Galemys pyrenaicus*, genetic, microsatellites, monitoring

INTRODUCTION

The Pyrenean desman *Galemys pyrenaicus* (Geoffroy St. Hilaire, 1811) (Chordata, Mammalia) is a small semi-aquatic mammal endemic to the Pyrenean Mountains and the northern half of the Iberian Peninsula where it lives in mountain streams of cold and well-oxygenated flowing waters (Nores et al., 2007). This species is considered as vulnerable in the IUCN Red List (Fernandes et al., 2008) and its ecology and biology are poorly known, notably because of its elusive behaviour and its primarily nocturnal activity (Stone, 1987a; Bertrand, 1994). However, scientists agree that this species, like other species living in mountain streams, has been suffering from fragmentation and habitat loss, due to the increase of human impact on nature. An example of the human impact on rivers is the construction and operation of hydroelectric power plants. These can lead to physical and biotic modifications and they can alter both hydrologic and thermal regimes, impacting on the resources of benthonic larvae

of macro-invertebrates (QUEIROZ et al., 1992; CÉRÉGHINO & LAVANDIER, 1997). Although little information is available for the Pyrenean desman, these detrimental effects have been studied on other mammals and birds, and highlighted by various authors (NILSSON & DYNESIUS, 1994; D'AMICO et al., 2000). In France, the Aude River has been deeply impacted by the hydroelectric plant of Nentilla, and particularly by its recent repair, which induced modifications of water flows. The monitoring of Pyrenean desmans living in the section of the river downstream from the hydroelectric plant was therefore an opportunity to evaluate the potential impacts of these modifications on this species.

The presence of the Pyrenean desman can be essentially detected by two methods: faeces sampling and live-trapping. These were tested and compared in previous studies (NORES et al., 1998; GONZÁLEZ-ESTEBAN et al., 2003). While live-trapping is onerous to implement and potentially a risk for the species, faeces sampling is non-invasive but should be undertaken with foresight. Specifically, some mistakes can easily occur when collecting samples. Indeed, the Pyrenean desman shares its habitat and diet with other semi-aquatic vertebrate species, such as the water shrew (*Neomys fodiens*) and the white-throated dipper (*Cinclus cinclus*), and therefore faeces can be confounded when they are not fresh or have been in contact with water. However, a recent reliable and non-invasive method was developed to easily distinguish faeces of the Pyrenean desman from these other species (GILLET et al., 2015a). Moreover, genotyping of faeces with microsatellite markers can be used to survey populations or to monitor individuals of rare mammal species (TABERLET et al., 1997; EGGERT et al., 2003; MONDOL et al., 2009a, 2009b). Therefore, we monitored individuals of Pyrenean desmans living in the section of the Aude River impacted by the Nentilla hydroelectric plant by combining the genotyping of faeces samples and hairs of live-trapped animals.

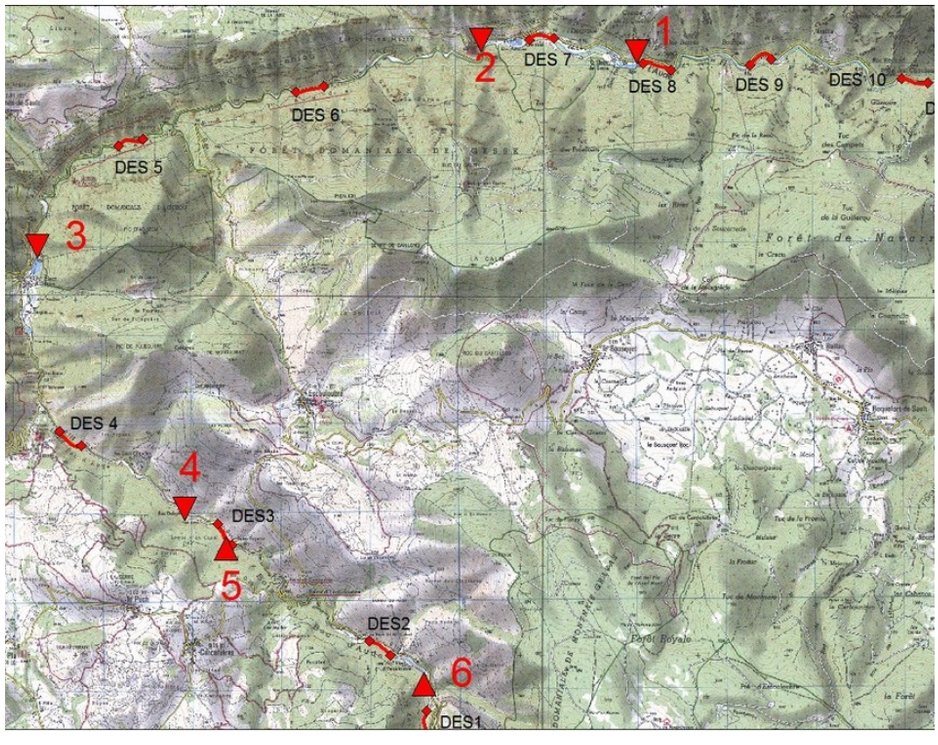

Fig. 1. – Map of the study area showing the ten river segments (DES1 to DES10) and different obstacles (red arrows) along the Aude river. All obstacles are water intakes of different heights: 3m, 1m, 9m, 1m, 75cm and 2m for obstacles 1 to 6, respectively.

MATERIAL AND METHODS

Sampling protocol

Three campaigns of faeces sampling were conducted: between January and April 2011, between March and July 2012 and between July and August 2013 respectively. The dates were not fixed since faeces sampling was dependent on the weather and the river flows. Along 20 km of the Aude River, ten 300m-long segments were surveyed twice a year (Figure 1). These segments of the Aude River were situated in the Department of the same name, in the eastern part of the French distribution of the Pyrenean desman. The mean elevation of these segments was 693 m with the highest segment being DES 1 (990 m) and the lowest DES 10 (455 m) (Figure 1). Banks of the river mostly consisted of trees, shrubs and bushes.

During the first campaign, each faeces sample was collected in a small tube (1.5ml) fitted in a larger one containing silica-gel. During the second and third campaigns, faeces were collected in tubes (1.5ml) containing absolute ethanol. The geographic position of each faeces was recorded on a GPS device.

Additionally, trapping sessions were conducted in three segments (DES3, DES7 and DES9, Figure 1) during six nights (two nights per segment) in September 2011, 2012 and 2013. Each trapping segment was extended to 900m long (or less depending on the accessibility to the river) and was divided in sub-segments of 300m long (or less). Eighteen traps, formed by two modified fish-traps hooked to each other, were set up in the most homogeneous way, without fixing arbitrary distances between them. Traps were laid where the strongest water flow occurred, in the river or near the banks, with the entrance facing the water flow and the rear portion out of the water to form a repository for the trapped Pyrenean desman. They were laid late in the day (18:00 local time) and removed early in the next morning (4:00 local time). Traps were checked every hour. Captured Pyrenean desmans were weighed, sexed, eventually marked with varnish on claws of a hind leg or with a transponder, and a hair sample taken that was immediately placed in a 1.5ml tube containing absolute ethanol. Hair samples were collected under licenses from the French Government: n° 2011221-0004 for Aude Department and n° 2011-INT/01 for Ariège Department. Animals were then released at the site of capture. A post-trapping sampling of faeces was achieved each time in the three segments.

Sampling and DNA extraction

Genomic DNA from hair and faeces samples preserved in ethanol was extracted using the QIAmp DNA Micro Kit (Qiagen Inc., Hilden, Germany) and the Stool Mini Kit (Qiagen Inc., Hilden, Germany), respectively, all following the manufacturer's instructions. To avoid any cross contamination, DNA extractions from faeces samples were conducted in a separated room with a UV-sterilised platform where no Pyrenean desman tissue samples were previously treated.

DNA amplification

Identification of Pyrenean desman from faeces was ascertained by amplification of a small cytochrome b fragment, as described in Gillet et al. (2015a). Then hair samples and faeces were genotyped at 24 variable microsatellite loci (Table 1). Microsatellites were distributed in five Multiplex kits based on size limitations. PCRs were carried out in 10µl volume containing 0.15 of each 20µM primers, 7.5µl of Multiplex PCR kit (Qiagen Inc., Hilden, Germany) and 5µl of DNA. Amplifications were performed in a thermal cycler VWR Unocycler using one activation step at 95°C for 15min followed by 35 cycles (denaturation at 94°C for 30 s, annealing at 57°C for 90 s, extension at 72°C for 60s) and final extension step at 72°C for 30 min. Amplified DNA was analyzed for length variations on an ABI 3700 sequencer using GeneScan 500LIZ®

TABLE 1

Genotyping of 24 microsatellite loci in Pyrenean desman of the Aude River (France). Null allele frequency (NAF), rates of positive PCR (PCR+), allelic dropout (ADO) and false allele (FA) for each locus.

Locus	H_E	H_O	No. alleles	NAF	PCR+	ADO	FA
GpyrGS06	0.10	0.06	2	0.13	0.77	0.00	0.009
GpyrGS07	0.04	0.03	2	0.00	0.74	0.00	0.000
GpyrGS10	0.31	0.31	4	0.00	0.77	0.06	0.013
GpyrGS11	0.17	0.17	3	0.02	0.78	0.07	0.000
GpyrGS12	0.10	0.05	2	0.00	0.82	0.00	0.009
GpyrGS13	0.13	0.03	2	0.10	0.89	0.00	0.000
GpyrGS18	0.12	0.10	2	0.00	0.80	0.00	0.000
GpyrGS20	0.57	0.38	4	0.00	0.74	0.00	0.014
GpyrGS22	0.29	0.24	3	0.07	0.82	0.21	0.000
GpyrGS23	0.43	0.48	3	0.00	0.79	0.05	0.000
GpyrGS30	0.13	0.07	2	0.00	0.77	0.00	0.000
GpyrGS32	0.62	0.41	3	0.00	0.81	0.17	0.000
GpyrGS33	0.29	0.17	2	0.20	0.74	0.10	0.000
GpyrGS34	0.38	0.41	3	0.00	0.79	0.26	0.000
GpyrGS41	0.07	0.07	2	0.00	0.92	0.21	0.000
GpyrGS46	0.05	0.05	2	0.12	0.87	0.00	0.000
GpyrGS47	0.07	0.03	2	0.08	0.87	0.00	0.000
GpyrGS53	0.03	0.03	2	0.16	0.87	0.00	0.000
GpyrGS55	0.08	0.03	2	0.00	0.83	0.00	0.000
GpyrGS74	0.04	0.03	2	0.09	0.84	0.00	0.000
GpyrGS75	0.06	0.06	2	0.07	0.87	0.00	0.000
GpyrGS80	0.12	0.09	2	0.00	0.88	0.00	0.000
GpyrGS82	0.49	0.21	2	0.15	0.47	0.25	0.014
GpyrGS94	0.07	0.07	2	0.00	0.93	0.00	0.000

size standard and alleles were scored on GENEMAPPER 4.0 (Applied Biosystems).

In order to avoid genotyping errors in our dataset, consensus genotypes had to be constructed for faeces samples. For this, we used a modified multitube PCR approach (TABERLET et al., 1996) and we repeated four times each PCR. We chose to accept scoring alleles if they appeared at least three times out of the four PCRs and we only considered 100% matches to accept identical genotypes.

Statistical analysis

Each replicate genotype was compared with the consensus genotype to quantify the error rates; both the construction of the consensus genotype and the quantification of error rates such as false alleles (FA) and allelic dropouts (ADO) were performed using GIMLET v1.3.3 (VALIÈRE, 2002). Expected (H_E) and observed (H_O) heterozygosity, as well as the probability of identity among siblings PIDsibs (i.e. the probability that two related individuals have

the same genotype (WAITS et al., 2001)) were estimated using GIMLET v1.3.3. The Hardy–Weinberg (HW) equilibrium was tested using the exact test implemented in GENEPOP 4.1.0 (Rousset, 2008). Tests for linkage disequilibrium between loci were performed using GENEPOP 4.1.0 and MICRO-CHECKER 2.2.3 (VAN OOSTERHOUT et al., 2004) was used to estimate the proportion of null alleles (NA).

RESULTS

A total of 131, 160 and 150 putative faeces of Pyrenean desman were collected in 2011, 2012 and 2013 respectively. In addition, 7, 2 (one new individual and one recapture) and 3 individuals were trapped for the same years, respectively (Table 2). Out of the 441 collected faeces, 267 were assigned to the Pyrenean desman, and one to the white-throated dipper, after sequencing a small cytochrome b fragment. DNA was probably too much degraded in the other faeces or these belonged to other species not targeted by our primers. 204 Pyrenean desman faeces (64, 80 and 60 in 2011, 2012 and 2013 respectively) were genotyped for microsatellite loci (Table 1). The mean H_O was 0.15 (ranging from 0.03 to 0.48) while the mean H_E was 0.20 (ranging from 0.03 to 0.62) (Table 1). The number of alleles ranged from 2 to 4 (Table 1). Tests for HWE showed significant deviations for only one locus (GpyrGS13, p-value = 0.001) and no linkage disequilibrium was found after Bonferroni's correction. The mean proportion of positive PCRs was 81%, ranging from 47% to 93% among loci and from 39% to 100% among samples. The mean Allelic Dropout (ADO) rate was 0.06, ranging from 0 to 0.26, and the mean false allele (FA) rate was 0.01, ranging from 0 to 0.014 (Table 1).

MICRO-CHECKER did not detect any significant bias in our dataset that could be attributed to null alleles (Table 1). A total of 28 additional individuals were identified from faeces with a PID (sibs) of $4.46e^{-03}$.

Nineteen individuals were identified in 2011, seven were trapped (four males and three females) and twelve were identified from faeces. Furthermore, two trapped animals were identified again from faeces in the same segments (DES3 and DES9), two and three days after their capture respectively. One individual, identified from faeces, was found 4.8 km upstream from DES9 (on May 3), in DES6 (on June 9).

Nine new individuals were identified in 2012, one (female) was trapped and eight were identified from faeces. In addition, one individual (female) trapped in 2011 was recaptured in 2012 (Table 2). Two animals identified in 2011 were identified again from faeces collected in 2012. The first one was found 16.2 km upstream from DES7 (on September 20, 2011), in DES1 (on July 13, 2012). The second was found 17.8 km upstream from DES9 (on September 20, 2011), in DES2 (on September 7, 2012).

Eleven new individuals were identified in 2013, three (females) were trapped and eight were identified from faeces. One animal identified in 2011 was identified again in 2013 in the same river segment (DES3). One of the three trapped individuals was identified from faeces collected two months before its capture. Finally, the short-term movement of one individual was evidenced, as it was found 1.6 km downstream from DES8 (on August 20), in DES9 (on August 28).

The number of individuals identified per river segment and per year is given in Table 1. The river segments DES3, DES7 and DES9 hosted 8, 9 and 11 individuals, respectively. No desman was found in three segments: DES5, DES10 and DES13.

DISCUSSION

According to our results, the genotyping of the 24 microsatellites proved to be a reliable technique to identify Pyrenean desmans and to implement individual monitoring as several animals were found one or two years after being

TABLE 2

Number of collected faeces / number of faeces belonging to the Pyrenean desman / number of genotyped faeces / number of identified individuals of Pyrenean desman (**in bold**), per river segment and per year in the Aude River (France). Trapping sessions occurred in segments DES3, DES7 and DES9. Numbers in brackets are trapped individuals that are included in the total number of identified individuals. "*" indicates one individual trapped in 2011 that was recaptured in 2012.

	DES1	**DES2**	**DES3**	**DES4**	**DES5**	**DES6**	**DES7**	**DES8**	**DES9**	**DES10**	**Total**
2011	3/3/3/**1**	0	14/13/13/**5 (1)**	0	9/0	6/0	42/16/14/**7 (3)**	8/5/5/0	42/29/29/**6 (3)**	7/0	19
2012	53/37/37/**2**	38/27/27/**2**	5/3/3/0	1/1/1/0	14/0	11/4/4/**2**	7/4/4/**1**	11/1/1/0	6/2/2/**3 (1) (1*)**	14/1/1/0	9
2013	35/31/8/0	35/30/7/0	34/16/9/**3 (1)**	6/6/6/**1**	6/6/6/0	9/9/6/**2**	8/8/6/**1 (1)**	6/6/6/**1**	8/8/5/**3 (1)**	3/1/1/0	11
Total	3	2	8 (2)	1		4	9 (4)	1	11 (5)	0	39

first identified. However, the results of this technique are highly dependent on the freshness, and the size of faeces, at the time of collecting. Indeed, Pyrenean desman faeces are generally small (10 to 15mm long and 4 to 8mm wide, (Bertrand 1993)) and the contained DNA degrades quickly in nature due to water and UV radiation. Genotyping faeces can also be seen as complementary to the trapping of individuals as it is without risk of harm to the animals and requires less field workforce. On the other hand, the trapping of Pyrenean desmans can provide more information on the individuals such as their sex, size, body mass, etc.

The three, yearly sessions of faeces sampling and trapping successfully led to the identification of 39 individuals along the 20 km stretch of the Aude River. However, the number of individuals identified from faeces could be overestimated due to error rates. Eleven individuals were trapped: 4 males and 7 females. The other 28 individuals identified from faeces could not be sexed and therefore the sex-ratio could hardly be representative of the population. The highest number in a 300m segment of river was found in 2011 in DES7 (n = 7), followed by DES9 (n = 6) and DES3 (n = 5). However, these results could be impacted by the time of sampling and the water flows. Moreover, all collected faeces could not be genotyped and therefore the number of animals could be underestimated in other segments and/or years. As the length of the sampled river segments is within the order of magnitude of reported home ranges (Stone, 1987b; Silva, 2001; Gisbert & Garciá-Perea, 2004; Melero et al., 2012), these high numbers of Pyrenean desman per segment could support the results of Melero et al. (2012, 2014) concerning the social behaviour of this species and the overlapping of home ranges.

Movements of four individuals were evidenced during this study as they were found at distances of 1.6 km, 4.8 km, 16.2 km and 17.8 km, eight days, one month, ten months and one year later, respectively. Although several distances between rest sites have been reported (Melero et al., 2012), long-distance movements have never been recorded before for the Pyrenean desman. Stone (1987a) showed that populations of Pyrenean desman were composed of sedentary and erratic individuals, the latter being mainly juveniles and solitary adults. As the four individuals mentioned above were identified from faeces, we have no information on their sex or age. These movements could be related to modifications of the river during the repair works. Indeed, from November 2011, very high water flows have been observed downstream from the segment

DES2 and they have profoundly impacted the river bed. Therefore some individuals may have moved upstream to find more suitable habitat, notably in the less impacted segments DES1 and DES2. This also means that the Pyrenean desman can disperse when its habitat is impoverished and that its response can be quick. In addition, individuals who moved over long distances should have encountered hydraulic plants or dams on their way (Figure 1). This could confirm that the Pyrenean desman is able to cross over such obstacles, as it was previously suggested (Chora & Quaresma, 2001).

CONCLUSION

This study confirms the genotyping of faeces as a reliable method to identify Pyrenean desmans, in addition to, or as a substitute for trapping, in order to perform individual monitoring. However, sexing the animals from faeces should be investigated, while ageing will remain impossible, making difficult direct studies of population dynamics. Several movements of individuals evidenced that Pyrenean desmans can travel long distances. This new and unexpected finding is particularly interesting in terms of conservation. However, a longer term study is required to investigate the reason for this dispersal. Finally, the observed numbers present in short segments demonstrate that the Aude River, and its basin, are suitable habitat for the Pyrenean desman, and emphasise that particular attention should be paid to this river in regard to conservation of this species.

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SHORT NOTES

An ecophysiological discussion of trace element bioaccumulation in cultured *Mytilus galloprovincialis*

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Monitoring programs conducted by the French Research Institute for the Exploitation of the Sea IFREMER have been using the mussel watch approach introduced by Goldberg [1] since 1974, initially on wild and cultured bivalve mollusks [2], leading to long time data series for several trace elements (TEs: Ag, Cd, Cr, Cu, Hg, Ni, Pb, V and Zn; http://envlit.ifremer.fr/). Since 1996, transplanted caged *Mytilus galloprovincialis* LAMARCK, 1819 have been used to characterize the chemical contamination of Mediterranean coastal waters even in locations where no native wild mussels were available. This project succeeded in assessing the natural background and the extent of the chemical contamination first at the scale of the French Mediterranean littoral [3,4], and more recently at the scale of the whole western Mediterranean Sea [5,6]. However, these programs have focussed on a limited number of metals. Nowadays, the development of very sensitive equipment allows the measurement of some TEs found at very low environmental levels. In parallel, recent technological developments have led to an increase in the extraction and industrial refinement of TEs previously of little concern. Therefore, the environmental monitoring of less studied, potentially toxic TEs of emerging environmental concern is relevant [7].

From data previously published by RICHIR & GOBERT [7], the first objective of this short note was to discuss the bioaccumulation profile of 19 TEs that have been either broadly (Cr, Ni, Cu, Zn, Cd, Pb, As, Ag and V) or little monitored (Be, Al, Fe, Mn, Co, Se, Mo, Sn, Sb and Bi) in the Mediterranean mussel *M. galloprovincialis*. The second objective was to test the relevance of the Trace Element Pollution Index of RICHIR & GOBERT [8] when modelling the effect of the shell length and flesh dry weight on the overall accumulation of these 19 TEs in rope-grown mussels. Because of the importance of gametogenesis in the physiological cycle of *M. galloprovincialis*, the third objective was to briefly discuss the deterministic effect of the sex and the reproductive status on the overall TE bioaccumulation and the TE-specific compartmentalization in that species.

Briefly, to realise these three objectives, *M. galloprovincialis* were purchased from the shellfish farm of the Diane pond on the eastern coast of Corsica, France (42°07'45.00''N, 9°31'01.00''E), in March 2010 (after mussel spawning) and February 2011 (before mussel spawning). Seventy four mussels sampled in February 2011 were used to investigate the bioaccumulation of the 19 TEs listed above. These 74 mussels were further segregated according to their sex to study differences between male and female TE bioaccumulation prior to spawning. Forty supplementary large-size (70-80 mm shell length) mussels purchased in March 2010

(n = 20) and February 2011 (n = 20) were used for the analysis of body compartmentalization after and before spawning, respectively, at one-year interval. Body compartments were sorted as follows: gills, hepatopancreas, mantle and remaining soft tissues. TE levels were determined by ICP-MS (ICP-MS ELAN DRC II, PerkinElmer®) after mineralization in a closed microwave digestion labstation (Ethos D, Milstone Inc.), using HNO_3 and H_2O_2 as reagents (Suprapur grade, Merck). Analytical accuracy was checked by analysing Certified Reference Materials: BCR 278, NIST 1566b and NIST 2976. The global mean recovery, all elements together, was 95 ± 9 %. For each TE, the analytical detection limit (L_D) was calculated according to CURRIE [9] or GRINZAID et al. [10].

The Trace Element Pollution Index (TEPI) of RICHIR & GOBERT [8], which synthetizes the accumulated levels (concentrations or contents) of all the studied TEs into a single index value, was calculated for each of the 74 mussels sampled in February 2011 as follows: $TEPI = (Cf_1 * Cf_2 \ldots Cf_n)^{1/n}$, where Cf_n is the mean normalized concentration or content of the TE n of a given mussel [11,12]. The log-transformed power function: $\log_{10}Y = \log_{10}a + b\log_{10}X$, and the linear regression: $Y = bX + a$, were tested to model the relationships between the 74 mussel flesh dry weight (X; from 0.17 to 3.36 g) or shell length (X; from 43.40 to 86.41 mm) and TEPI values (Y) [13,14,15]. *b* is the slope of linear functions; $\log_{10}a$ and *a* are the Y-intercepts. To select the most adequate model that best described these relationships, the second order Akaike information criterion (AICc) was used [16]. Statistical analyses were performed with STATISTICA 10 (Stat-Soft Inc.) and GraphPad Prism 5 (GraphPad Software Inc.) software.

Results showed that rope-grown *M. galloprovincialis* from the Diane pond efficiently bioaccumulated the 10 little monitored TEs (Be, Al, Fe, Mn, Co, Se, Mo, Sn, Sb, Bi) in addition to the 9 TEs classically monitored (Cr, Ni, Cu, Zn, Cd, Pb, As, Ag and V) in that species (Table 1). Comparative graphs ordering TEs either broadly or little monitored in *M. galloprovincialis* by decreasing order of concentrations are given in Fig. 1. Concentrations ranged from 10^{-3} µg g_{DW}^{-1} for Bi to 10^{2} µg g_{DW}^{-1} for Al and Fe (Table 1; Fig. 1). Essential TEs classically monitored such as Zn, Cu, Ni and Cr appeared to be preferentially accumulated unlike non-essential toxic TEs such as Cd, Pb and Ag (Fig. 1a) [17]. With regard to As, this TE was reported to be naturally more bioaccumulated in *M. galloprovincialis* [3], mostly as organicated non-toxic species [18,19]. The mean V concentration was relatively high compared to data reported by the IFREMER (http://envlit.ifremer.fr/) for the Diane pond and could thus reflect a temporary moderate contamination of the pond by that specific element [7]. Bioaccumulation of TEs little monitored in *M. galloprovincialis* showed a similar graphic profile (Fig. 1b) to the previous one. Environmentally abundant and/or essential TEs such Fe, Al, Mo, Mn, Se and Co were bioaccumulated in a more important way, while concentrations of non-essential and potentially toxic Sn, Be, Sb and Bi remained low to very low [17]. The Diane pond has previously been considered little contaminated by TEs [7]; Fig. 1 thus presents the natural aptitude of rope-grown *M. galloprovincialis* to bioaccumulate TEs in clean environmental conditions. Although essential TEs appeared to be preferentially accumulated, unlike non-essential toxic ones, there is currently little physiological evidence about this preferential accumulation of essential versus other elements [20,21].

The overall concentration of the 19 studied TEs in mussel flesh, synthetized as TEPI values, decreased when the mussel flesh dry weight increased (Fig. 2a); their overall flesh content increased when the mussel shell length increased (Fig. 2b). According to AICc values, the relationships between the overall TE concentration of the 74 mussels, without size restriction, and their body dry weight, or between the overall TE content and the mussel shell length, were better modelled by the power function (Fig. 2a; Table 2a; AICc value of 93.08%) or the linear regression (Fig. 2b; Table 2b; AICc

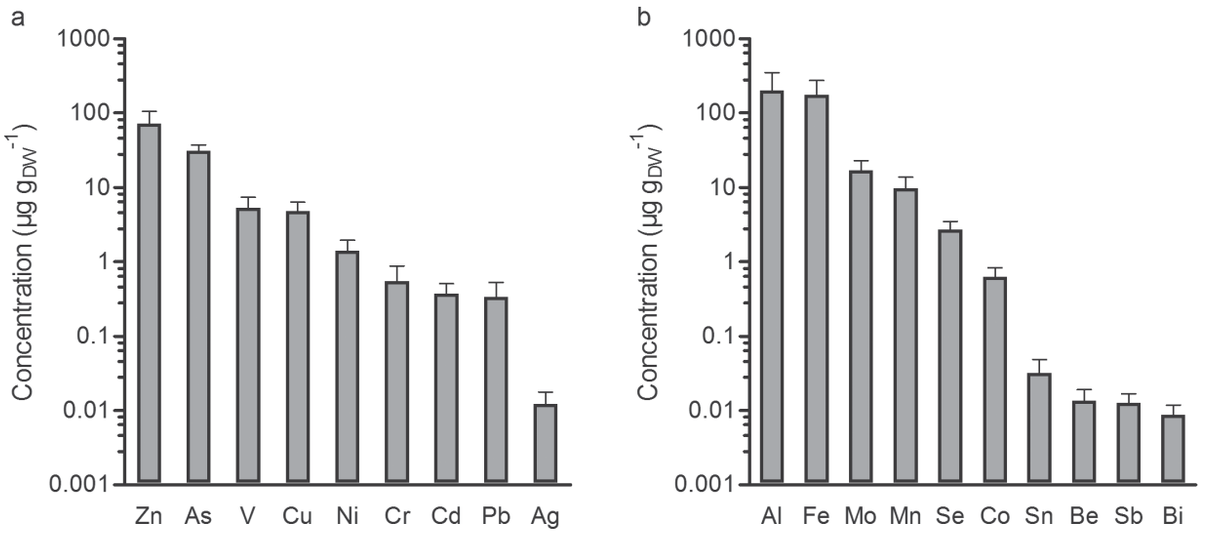

Fig. 1 – Concentration profiles (mean ± SD, in µg g_{DW}^{-1}; logarithmic scale) of trace elements (TEs) either (a) broadly or (b) little monitored in *Mytilus galloprovincialis* (n = 74).

value of 96.47%), respectively. In contrast, the relationship between the overall TE content and the mussel shell length was properly modelled by both the linear regression and the power function when only individuals larger than 55 mm were considered (Table 2b; AICc values of 47.90% and 52.10%). Small-size mussels (< 55 mm) thus had an antagonistic effect on the modelling of the overall TE bioaccumulation in rope-grown *M. galloprovincialis*: they led us to elect the linear regression to model the relationship between the mussel size and their overall TE content (AICc value rising from 47.90% to 96.47%), but sensibly diminished the significance of the power function modelling the relationship between the mussel flesh dry weight and their overall TE concentration (AICc value decreasing from 98.83% to 93.08%). These results corroborate the observations of Richir & Gobert [7] made for each TE considered separately and support

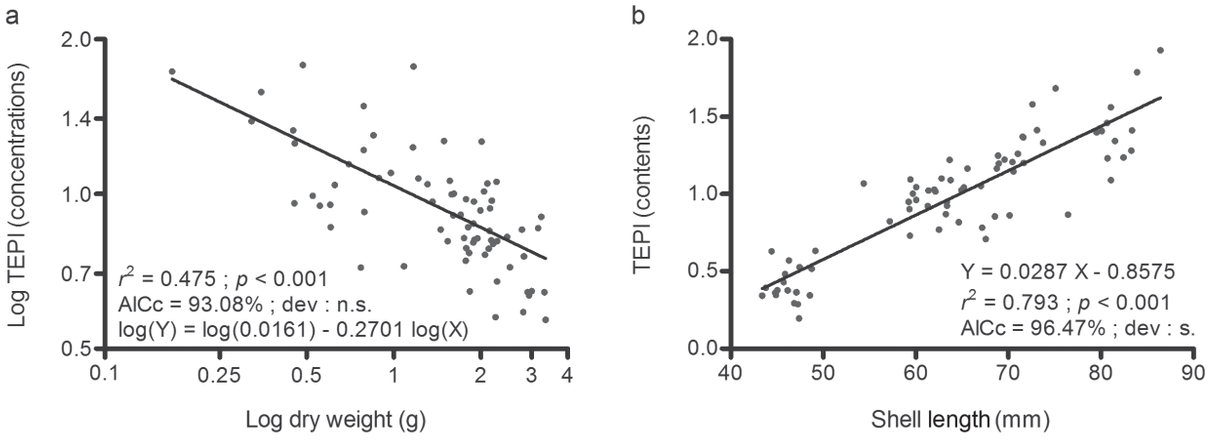

Fig. 2 – (a) Log transformed power function modelling the relationship between *Mytilus galloprovincialis* (n = 74) soft tissue dry weight and Trace Element Pollution Index (TEPI) values (no unit), calculated from mean normalized concentrations of the 19 studied trace elements (TEs), and (b) linear regression modelling the relationship between mussel shell length and TEPI values (no unit), calculated from mean normalized contents of the 19 studied TEs. Linear equations and their corresponding fitting parameters (r^2; p-value; deviation (dev.) from the model: s = significant, n.s. = non-significant; AICc) are reported on graphs.

TABLE 1

Trace element (TE) concentrations (mean ± SD, in µg g_{DW}^{-1}) in rope-grown *Mytilus galloprovincialis* purchased before they spawned, and Trace Element Pollution Index (TEPI) values (no unit) calculated from mean normalized concentrations of the 19 studied TEs. TE concentrations and TEPI values are given for all mussels together, independently of their size or sex, for mussels sorted by size-class (cl. 1 to cl. 4) and for mussels sorted by sex. Numbers between brackets are numbers of mussels. Letters represent significant differences ($p < 0.05$) between size-classes; * represent significant differences ($p < 0.05$) between sexes. Concentration data used to calculate TEPI values are from RICHIR & GOBERT [7].

	All mussels (74)	**Mussels sorted by size-class**								**Mussels sorted by sex**			
			cl. 1: 43-54 mm (19)		cl. 2: 55-64 mm (21)		cl. 3: 65-74 mm (20)		cl. 4: 75-87 mm (14)		Females (29)		Males (45)
Al	200 ± 150	a	323 ± 223	ab	162 ± 63	b	150 ± 95	ab	160 ± 88		204 ± 143		197 ± 156
V	5.35 ± 2.02	ab	5.80 ± 2.50	a	5.67 ± 1.84	ab	5.43 ± 1.67	b	4.11 ± 1.68	*	6.55 ± 2.40	*	4.57 ± 1.24
Fe	177 ± 97	a	255 ± 146	ab	156 ± 43	b	148 ± 63	b	146 ± 55		186 ± 89		172 ± 102
Cr	0.554 ± 0.320		0.803 ± 0.489		0.477 ± 0.146		0.462 ± 0.197		0.465 ± 0.181		0.581 ± 0.288		0.537 ± 0.341
Mn	9.86 ± 3.87	a	12.89 ± 4.03	b	9.84 ± 2.84	b	9.07 ± 3.62	b	6.88 ± 2.51	*	12.18 ± 3.48	*	8.36 ± 3.37
Co	0.634 ± 0.205		0.688 ± 0.255		0.605 ± 0.135		0.626 ± 0.235		0.616 ± 0.173	*	0.707 ± 0.215	*	0.587 ± 0.185
Ni	1.41 ± 0.54	a	1.81 ± 0.66	b	1.30 ± 0.37	b	1.34 ± 0.50	b	1.16 ± 0.34	*	1.68 ± 0.59	*	1.24 ± 0.43
Cu	4.82 ± 1.50	a	5.55 ± 1.40	a	4.86 ± 1.47	a	4.98 ± 1.55	b	3.56 ± 0.69	*	6.50 ± 0.67	*	3.74 ± 0.61
Zn	72.6 ± 33.6		79.7 ± 37.8		66.2 ± 22.2		75.7 ± 43.8		67.9 ± 25.2	*	86.3 ± 36.4	*	63.7 ± 28.8
Se	2.70 ± 0.78	a	3.24 ± 0.66	ab	2.64 ± 0.76	b	2.64 ± 0.81	b	2.17 ± 0.48	*	3.48 ± 0.34	*	2.21 ± 0.54
Ag	0.0123 ± 0.0054	a	0.0157 ± 0.0068	a	0.0124 ± 0.0040	ab	0.0116 ± 0.0048	b	0.0083 ± 0.0027	*	0.0151 ± 0.0064	*	0.0104 ± 0.0038
Cd	0.374 ± 0.131		0.390 ± 0.200		0.389 ± 0.100		0.357 ± 0.089		0.352 ± 0.111	*	0.397 ± 0.106	*	0.358 ± 0.144
Sn	0.0318 ± 0.0167		0.0413 ± 0.0222		0.0282 ± 0.0117		0.0312 ± 0.0170		0.0248 ± 0.0073		0.0323 ± 0.0160		0.0314 ± 0.0174
Sb	0.0126 ± 0.0042		0.0152 ± 0.0052		0.0119 ± 0.0028		0.0119 ± 0.0040		0.0113 ± 0.0038	*	0.0140 ± 0.0048	*	0.0118 ± 0.0036
As	31.2 ± 6.1	a	32.7 ± 6.8	ab	31.7 ± 5.5	ab	32.3 ± 5.9	b	26.9 ± 4.5	*	36.3 ± 4.3	*	27.8 ± 4.6
Mo	17.1 ± 5.8	ab	16.3 ± 6.0	a	19.5 ± 4.9	a	18.8 ± 5.6	b	12.3 ± 4.2	*	20.7 ± 5.8	*	14.8 ± 4.5
Be	0.0135 ± 0.0056		0.0169 ± 0.0085		0.0122 ± 0.0030		0.0121 ± 0.0042		0.0128 ± 0.0040		0.0127 ± 0.0056		0.0140 ± 0.0057
Pb	0.336 ± 0.192		0.400 ± 0.253		0.268 ± 0.138		0.324 ± 0.185		0.369 ± 0.154		0.378 ± 0.211		0.309 ± 0.175
Bi	0.0087 ± 0.0032		0.0100 ± 0.0039		0.0089 ± 0.0032		0.0082 ± 0.0026		0.0073 ± 0.0021	*	0.0097 ± 0.0034	*	0.0080 ± 0.0028
TEPI	0.959 ± 0.269	a	1.137 ± 0.307	ab	0.932 ± 0.198	b	0.920 ± 0.270	b	0.813 ± 0.190	*	1.094 ± 0.274	*	0.872 ± 0.229

the relevance of the TEPI to model, in a reduced number of synthesis equations, the relationships between the overall levels of bioaccumulated contaminants and the physiology of organisms.

The same was concluded when modelling the relationship between the shell length and the overall TE concentration of the 19 studied TEs in mussel flesh. Thus, the overall TE concentration in mussel flesh was linearly correlated (p = 0.0002) with the shell length when considering all the 74 mussels, without size restriction (Fig. 3). This significant relationship was to be attributed mainly to small-size mussels (43–54 mm) whose TE-specific and overall mean concentrations were always higher than for the mid-size individuals (55-64 mm and 65-74 mm), except for Mo, As and Cd (Table 1). Small-size mussels further showed a high inter-individual variability of their TE concentrations. These observations reflected unfavourable physical conditions of growth of small-size mussels found inside the rope [7]. When the linear regression model was run again for mussels larger than 55 mm only, the overall TE concentration in mussel flesh was no longer correlated (p = 0.1085) with the shell length (Fig. 3). For mid- to large-size *M. galloprovincialis* grown on ropes, the size did not significantly influence flesh concentrations of most TEs, although they were slightly lower on average in individuals larger than 75 mm (Table 1). As their culture begins synchronically, all mussels on a rope have the same age, but may differ in size according to individual growing conditions [14]. Thus, when sorting mussels for monitoring purposes, care should be taken to use neither small- (restrained growth and concentration effect) nor large-size (rapid growth and dilution effect) mussels, these individuals being not representative of the rope population [7].

Female and male mussels accumulated TEs unevenly during gametogenesis. As a result, mean TE concentrations in mussel dry flesh sampled prior to spawning differed significantly (p < 0.05) between sexes and were higher in females (from 3% for Al up to 74% for Cu, except for Be), for an overall difference (*i.e.* TEPI values) of 25% (Table 1). This different accumulation between individuals of opposite sexes resulted in the linear regression with slope still close to the significance threshold level of 0.05 when modelling the relationship between the shell length of mussels larger than 55 mm and their overall TE concentration (p = 0.1085; Fig. 3). The sex-related bioaccumulation of TEs during gametogenesis could depend on a functional role played by metallothioneins (MTs), as already suggested by Latouche & Mix [22] in the early 80s and supported by several subsequent experimental and field studies [23, 24, 25].

In *M. galloprovincialis*, up to 45 % of soft tissue weight can be lost during spawning [8]. Despite the importance of gametogenesis in the physiological cycle of *M. galloprovincialis*, the proportional distribution of TEs between main body compartments analysed respectively a few days before or after spawning, at one-year interval, remained the same: the 19 studied TEs were more accumulated in the hepatopancreas compared to the mantle and the gills (except for Mo in mussel having spawned), and only Zn, Se, Cd (at both reproductive states) and Mn (in mussels close to spawning) showed higher contents in the remaining soft tissues of mussels (Fig. 4). This very conservative character of TE compartmentalization (physiological and temporal constancy) is an argument in favour of some internal regulation of TE redistribution processes between organs [20,21], in addition to passive diffusion processes according to concentration gradients and tissue affinities.

To conclude, little monitored TEs as well as broadly monitored ones were efficiently bioaccumulated in rope-grown *M. galloprovincialis*, with a preferential accumulation for essential and abundant ones. The relevant use of the TEPI to model, in a reduced number of synthesis equations, the relationships between the overall levels of bioaccumulated contaminants and the physiology of organisms was described. The significant effect of the size of cultured mussels whose growth was above or below average on the accumulation of TEs in their flesh was pointed

TABLE 2

Comparison of linear regressions and log transformed power functions modelling (a) relationships between *Mytilus galloprovincialis* soft tissue dry weight and Trace Element Pollution Index (TEPI) values, calculated from mean normalized concentrations of the 19 studied trace elements (TEs), and (b) relationships between mussel shell length and TEPI values, calculated from mean normalized contents of the 19 studied TEs. Modelling was applied for all mussels together (n = 74) or for individuals large than 55 mm only (n = 55). *b* is the slope of linear functions; *a* and $\log_{10}a$ are the Y-intercepts. Fitting parameters are also indicated (r^2; *p*-value; deviation (dev.) from the model: s. = significant, n.s. = non-significant; AICc).

(a) Relationships between the overall TE concentration and the mussel flesh dry weight

	linear regression			log transformed power function	
	all mussels	mussels > 55 mm		all mussels	mussels > 55 mm
b	-0.2140	-0.2415	*b*	-0.2701	-0.5296
a	1.3190	1.3900	$\log_{10}a$	0.0161	0.0986
r^2	0.437	0.443	r^2	0.475	0.526
p	< 0.001	< 0.001	*p*	< 0.001	< 0.001
dev.	n.s.	n.s.	dev.	n.s.	n.s.
AICc	6.92%	1.17%	AICc	93.08%	98.83%

(b) Relationships between the overall TE content and the mussel shell length

	linear regression			log transformed power function	
	all mussels	mussels > 55 mm		all mussels	mussels > 55 mm
b	0.0287	0.0240	*b*	1.8530	1.4530
a	-0.8575	-0.5227	$\log_{10}a$	-3.3670	-2.6180
r^2	0.793	0.527	r^2	0.774	0.528
p	< 0.001	< 0.001	*p*	< 0.001	< 0.001
dev.	s.	n.s.	dev.	s.	n.s.
AICc	96.47%	47.90%	AICc	3.53%	52.10%

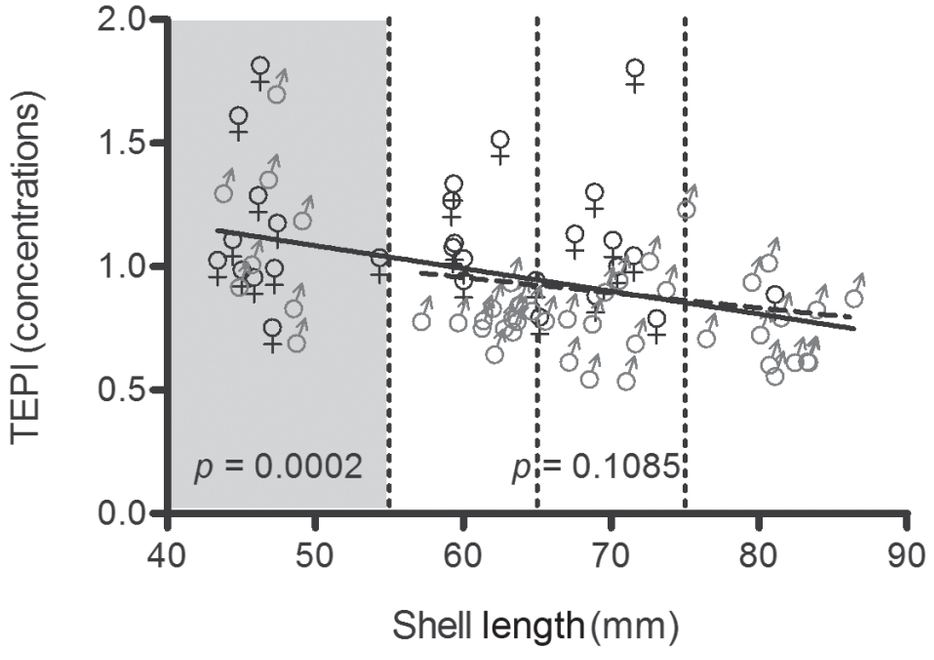

Fig. 3 – Linear regressions modelling the relationship between *Mytilus galloprovincialis* shell length and Trace Element Pollution Index (TEPI) values (no unit), calculated from mean normalized concentrations of the 19 studied trace elements (TEs), all mussels together (n = 74, shaded area included; full regression line) or restricted to mussels larger than 55 mm (n = 55, shaded area excluded; dashed regression line). Mussels were purchased before they spawned. ♀ and ♂ symbolize females and males, respectively. The dotted vertical lines separate mussels into 4 equivalent size-classes of about 10 mm. Model *p*-values (significant for all mussels together only) are given on the graph.

out, as was the effect of the sex of mussels close to spawning. Finally, the conservative character of TE compartmentalization regardless of the physiological status of sampled mussels, and their proportional redistribution between tissues, were suggested to rely on some internal regulatory processes that require further investigations.

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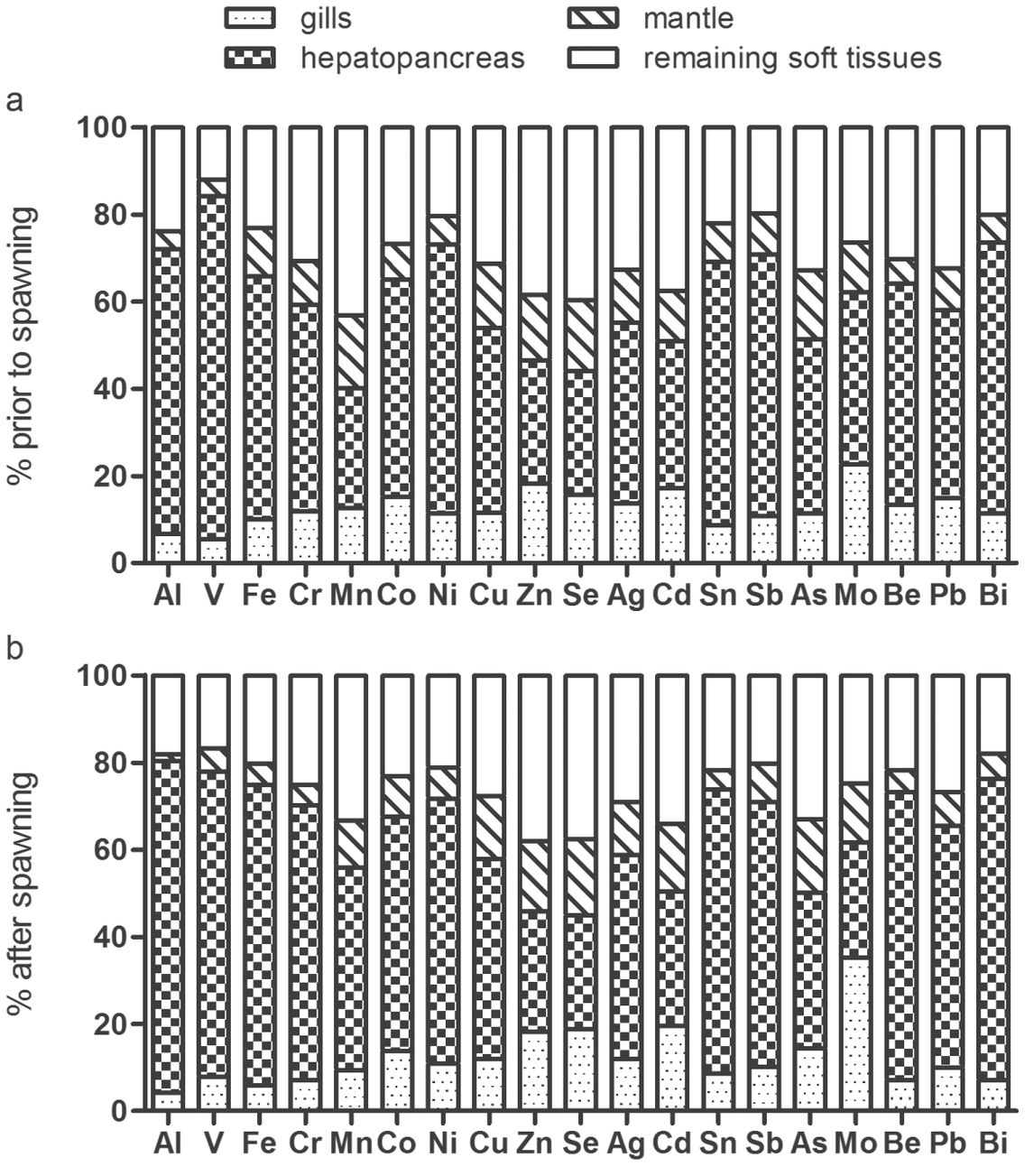

Fig. 4 – Proportional distribution of trace elements between main body compartments (gills, hepatopancreas, mantle and remaining soft tissues; in % of total contents) of *Mytilus galloprovincialis*. Mussels were purchased (a) a few days before they spawned (n = 20) and (b) a few days after they spawned (n = 20), at one-year interval.

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First offshore observation of parti-coloured bat *Vespertilio murinus* in the Belgian part of the North Sea

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KEY WORDS: parti-coloured bat, *Vespertilio murinus*, Belgian part of the North Sea

On September 19th, 2014 at 1:34am, a call sequence of a parti-coloured bat *Vespertilio murinus* was registered at approximately five kilometers off the Belgian coastline (N 51°21.40, E 3°6.06). This is the first observation of the species in the Belgian part of the North Sea. The meteorological conditions during the time of the recording were good, with a temperature of 18.6°C, a moderate easterly wind (3 Bft) and no precipitation.

The parti-coloured bat was detected from the Belgian research vessel 'Belgica' equipped with an automated acoustic SM3BAT recorder (wildlife acoustics Inc., Massachusetts, USA). The device records the echolocation calls of bats (between 0 and 126 kHz), from shortly before sunset to shortly after sunrise, hence allowing study of the spatio-temporal distribution patterns of bats in the Belgian part of the North Sea. The recordings were processed with the software programs SonoChiro (version v3.3.2; Biotope, France) and Batsound (version v1.3.1; Pettersson Elektronik, Sweden) to extract the echolocation calls of bats and to aid the identification to the species level. Every registration has a timestamp that is linked to the time and GPS registration of the ship, allowing determination of the exact time and location of observation.

The acoustic detector was operational during 84 nights in autumn 2014 (from 11 August until 5 December) and 76 nights in spring/summer 2015 (from 16 March until 22 July). This record was the only detection of a parti-coloured bat during this study.

The parti-coloured bat was registered for a period of 20 seconds, during which the call was recorded 60 times. This suggests the specimen was not just passing by the vessel. The call sequence is typical for an individual crossing an open biotope but some foraging calls (or buzz) are also noticeable. The intense activity suggests the parti-coloured bat was attracted by the vessel while passing by, where it most likely foraged for a short period of time.

The sonogram of the registered parti-coloured bat call shows an average frequency of maximum energy at 25.4 (± 0.30 standard deviation) kHz and an average duration of a single call of about 20 (± 1) ms (Fig. 1, Table 1), which is typical for the parti-coloured bat [1,2]. The rate of repetition of the calls is known to be about 5 Hz [1], which fits our recording.

The parti-coloured bat is a Palearctic species occurring over an extensive geographic area [1]. It is widely distributed from the Asiatic Pacific coast through Siberia to eastern, northern and central Europe in the west [2]. The western limit of its regular distribution lies in eastern France,

TABLE 1

Average, standard deviation, maximum and minimum values of the key variables of the 21 most distinctive calls in the sequence of the registered parti-coloured bat specimen. FI = initial frequency; FT = end frequency; FME = frequency of maximum energy.

	FI (kHz)	FT (kHz)	FME (kHz)	Call duration (s)	Intervals between two calls (s)
average	27.45	23.09	25.41	0.020	0.231
standard deviation	0.91	0.23	0.30	0.001	0.07
maximum	30.42	23.92	25.83	0.027	0.48
minimum	24.30	22.76	23.88	0.0177	0.10

Switzerland and southern Scandinavia (Fig. 2) [1]. The species is rare or absent in western and southern Europe, however vagrants have been recorded far outside its normal range, e.g. Tuscany [3], Spanish Pyrenees [4], Isle of Arran [5], Shetland Islands [5] and even oil rigs in the North Sea [6]. The species is known to migrate long distances of up to 2000 kilometers from Scandinavia and Central Europe to more temperate regions of Western Europe, and back [7,8,9]. Observations at the western limit of its distribution are linked to autumn dispersion and probably also hibernation [10]. For more information about the ecology of *Vespertilio murinus,* we refer to RYDELL & BAAGØE (1994) [1].

In Belgium, the parti-coloured bat has until now been recorded 41 times, including eight in 2014. The majority of those records were made in September and October, but there are also records in Belgium from February, March, April and June. The first observation was made in 1989 in the coastal community Blankenberge. A second record was made in the year 2000, and from 2006 onwards there is an increase in the number of records. Nine records were made with an acoustic detector, other records concern found (dead or injured individuals) or captured specimens or were sightings at a roosting location. Only three of the eight observations in 2014 were recorded by a detector. Most of the

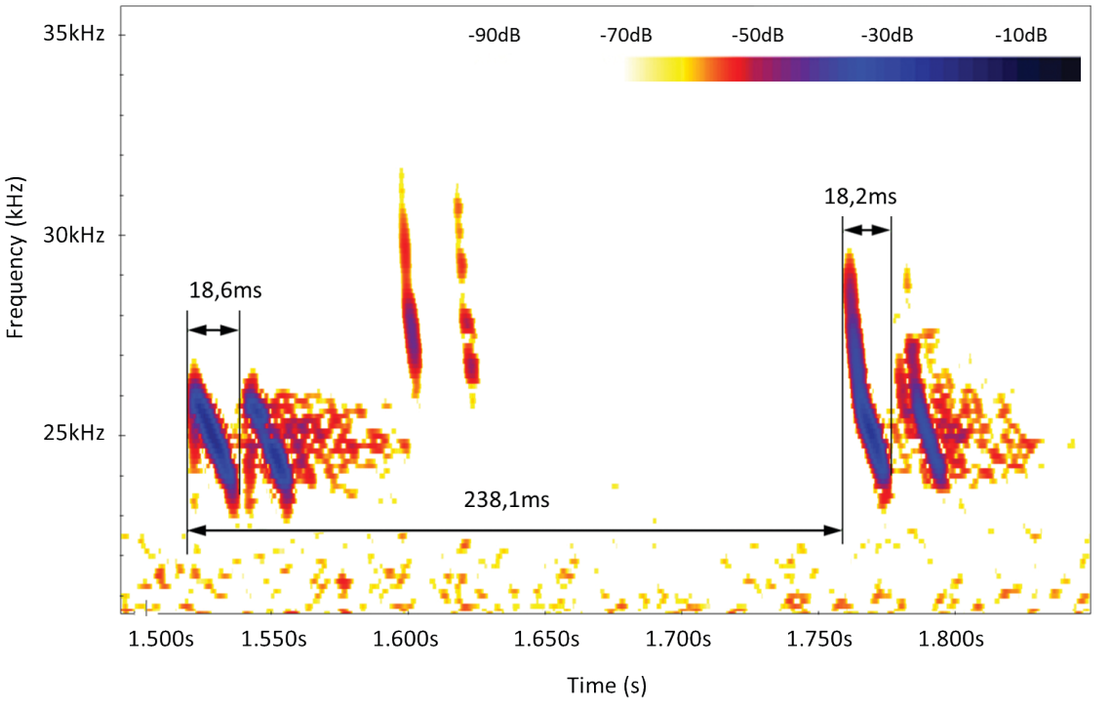

Fig. 1. – Sonogram of the parti-coloured bat *Vespertilio murinus* detected in the Belgian part of the North Sea.

Belgian observations were made onshore along the coast and along large riverbeds [11]. Similar to the Belgian records, 11 of the 24 observations of this species in the Netherlands between 1977 and 1995 were obtained from coastal localities [12]. Forty specimens were detected near the Swedish coast in 2005, 2006 and 2008 [13,14]. The parti-coloured bat has previously been detected offshore in the North and Baltic Seas. Three specimens were found on oil rigs in the Dutch part of the North Sea, once in 2004 and twice in 2006, at 69, 48 and 124 km from the coast [6]. Our observation hence contributes to this data series with increasing numbers of offshore observations. The species was, however, not recorded during an extensive study in the Dutch part of the North Sea [15], stressing the rarity of offshore observations as yet.

Our record of the parti-coloured bat at sea and other recent observations in Belgium may be considered indicative of an increased number of specimens in our region. Together with the reported records of specimens in e.g. the Spanish Pyrenees [4] and Italy [3], this suggests the species is extending its range of distribution to the West. The IUCN red list [16] also reports expanding populations in some part of the species' distribution range, for example in Denmark and the Netherlands. On the other hand, the contribution of improved methods of detection (e.g. acoustic techniques) and an increased search effort (both in time and space) also very likely contributed to the increased number of detections of the parti-coloured bat. For the Belgian records, this is, however, unlikely as only nine of the in total 41 records were done with an acoustic recorder. Of the eight records from 2014, only three were made with an acoustic detector. More extensive studies are indispensable to any unambiguous conclusion on the real status of the parti-coloured bat in south-western Europe.

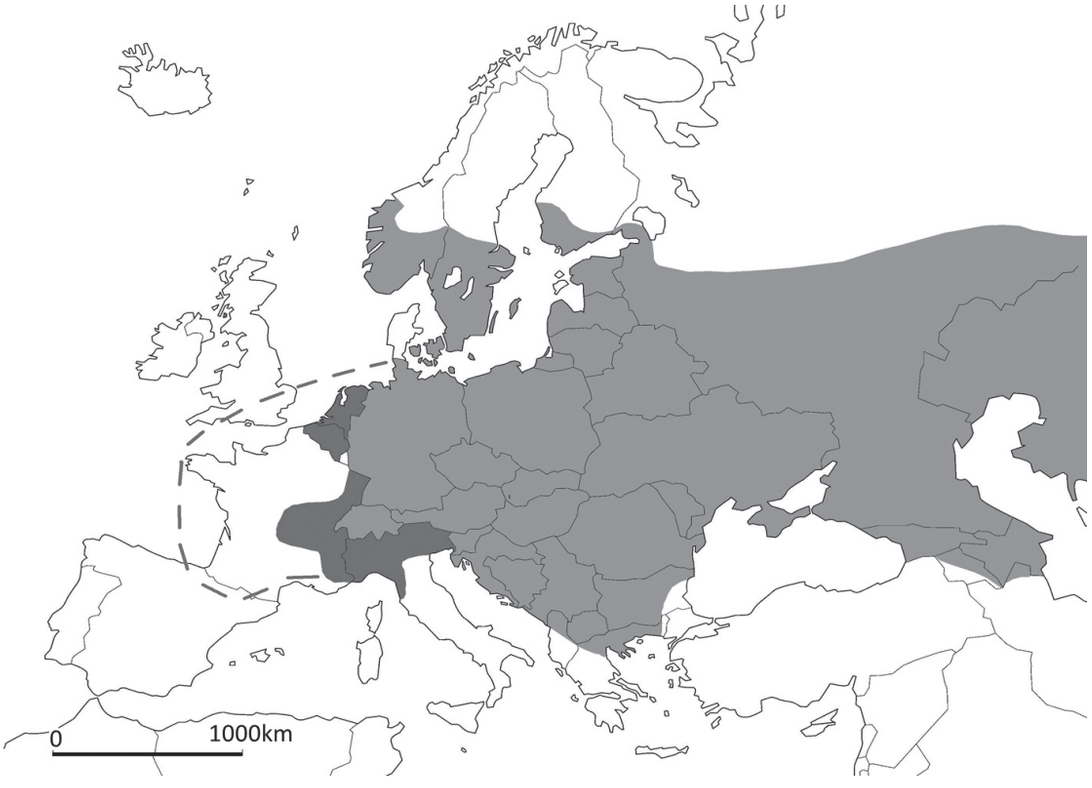

Fig. 2. – Distribution range of the parti-coloured bat *Vespertilio murinus* with indication of its breeding area (light grey) and its migration area (dark grey). The dotted line indicates the area where migrating individuals have been observed (adapted from ARTHUR & LEMAIRE 2015 [10] and www.eurobats.org [2]).

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A persistent population of the chocolate-band snail *Eobania vermiculata* (Gastropoda: Helicidae) in Belgium

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Eobania vermiculata (O.F. MÜLLER, 1774) is a large land snail species, with a maximum shell width of 33 mm. The species occurs in a variety of habitats, usually in dry vegetation, in hedgerows, gardens, vineyards and agricultural fields, often in coastal areas. Reproduction takes place in autumn. About 60 to 80 eggs are laid in the soil. The snails reach maturity two years after hatching. Adult snails hibernate in a hole in the soil and develop an epiphragma. Juveniles usually hibernate under stones or leaves [1,2]. *Eobania vermiculata* is a circum-Mediterranean species. Its native range extends from Spain to Turkey in Europe and along the North-African coast at least from Morocco to Libya, although it is absent as a native species in the SE of the Mediterranean region. The species has been introduced into several European countries, including Germany, Hungary, and The Netherlands. Introduced populations also occur in the USA, Australia, Japan, South Africa, Egypt, Israel, Saudi Arabia, Jordan, and Iran [1-9].

On September 17th 2014 five empty shells of *E. vermiculata* were collected near the Zeebrugge harbour (51°20'16"N; 3°10'47"E) in Belgium. The locality at Zeebrugge is a steep, SE-facing sandy slope situated between a road and a railroad track used for container traffic. Vegetation consisted of short grasses (mainly *Elytrigia atherica*), herbs and young *Rubus caesius*. During an additional half hour search carried out by two persons on November 9th 2014 twenty living juvenile and adult *E. vermiculata* and > 40 empty shells were found at the same locality. Most living *E. vermiculata* were found amongst rubble, mainly wooden planks and plastic, and in drainage pipes. Approximately one year after the species was first recorded, on September 9th 2015, a follow-up survey was carried out during a conchological excursion with a group of twelve persons. A 15-minute search yielded a total of 144 live *E. vermiculata* including 15 juveniles, and 22 empty shells. Finally, on September 10th, an extra 409 adult individuals, 49 juveniles, and 45 empty shells were found by two persons during a 45 minute collecting effort. In 2015, most *E. vermiculata* were found scattered among the short vegetation. Several individuals had formed an epiphragma, which may suggest that they were going into hibernation. These finds constitute the first evidence of a persistent *E. vermiculata* population in Belgium.

Considering the close vicinity of the Zeebrugge harbour, container traffic is the most likely pathway of introduction. Container shipments are known to be an important means by which land molluscs can colonize areas outside their native range. Snails may attach to the exterior of containers or may unintentionally be transported along with ornamental plants or vegetables [10]. In The Netherlands, living *E. vermiculata* have been found on cauliflower imported from Italy [11]. *Eobania vermiculata* is also traded and transported for human consumption [12].

There had been doubts about the species' ability to survive the winters in a temperate climate [4], although a population in The Netherlands has

persisted for at least five years [13]. The low effort required to collect large quantities of living adult and juvenile *E. vermiculata* at Zeebrugge one year after the population was first discovered shows that the species is most probably capable of surviving the Belgian winters. Several other Mediterranean land molluscs proved to be ecologically flexible and have established persistent populations in Belgium, including *Lehmannia valentiana* [14] and more recently *Hygromia cinctella* [15].

Eobania vermiculata is considered a potentially invasive species [16]. The early detection of *E. vermiculata* in Belgium allows close monitoring of the small, introduced population and measures to be taken to prevent further spread. Eradication of potentially invasive species is considered the most effective measure in the early stages of invasion, when populations are small and localized [17]. Therefore, in 2015, all *E. vermiculata* collected at Zeebrugge were removed and euthanized. Additional eradication efforts will be carried out in the near future.

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