BJZ

Belgian Journal of Zoology

Published by the

KONINKLIJKE BELGISCHE VERENIGING VOOR DIERKUNDE
KONINKLIJK BELGISCH INSTITUUT VOOR NATUURWETENSCHAPPEN

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SOCIÉTÉ ROYALE ZOOLOGIQUE DE BELGIQUE
INSTITUT ROYAL DES SCIENCES NATURELLES DE BELGIQUE

Volume 145 (2)
(July, 2015)

Managing Editor of the Journal

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Freshwater Biology
Vautierstraat 29
B - 1000 Brussels (Belgium)

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ISSN 0777-6276

Cover photograph by Bob Vandendriessche: *Myotis alcathoe* von HELVERSEN & HELLER, 2001, see paper by NYSSEN P. *et al*., page 131.

Zoology 2015

www.zoology2015.nl

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Matthew Evans (http://www.sbcs.qmul.ac.uk/staff/matthewevans.html)
Hanna Kokko (http://www.ieu.uzh.ch/staff/professors/kokko.html)
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Belg. J. Zool., 145(2) : 84-94 July 2015

Selective top-down control of epiphytic biomass by amphipods from *Posidonia oceanica* meadows: implications for ecosystem functioning

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ABSTRACT. Mediterranean *Posidonia oceanica* meadows shelter an important biomass and biodiversity of amphipod crustaceans that graze on epiphytes. However, their actual significance for ecosystem functional processes is hard to estimate, due to the lack of adequate data. Here, a field microcosm-based inclusion experiment was used to test if three of the dominant taxa of the amphipod community (*Apherusa chiereghinii*, *Dexamine spiniventris* and *Gammarus* spp.) could exert top-down control on seagrass leaf epiphytes. Influence of amphipod activity on nutrient availability for the host species was also investigated. All grazer taxa significantly reduced biomasses of erect macroalgae and erect sessile animals present on leaves. None of them consumed encrusting epiflora or epifauna. This selective top-down control could have important implications for the structure of the epiphytic community on leaves of *P. oceanica*, which is one of the most diverse and abundant of all seagrass species. Grazing activity of all taxa caused higher N content of seagrass leaves, likely through amphipod excretion and/or sloppy feeding. Since *P. oceanica* meadows often grow in oligotrophic zones where plant growth can be nutrient-limited, this N enrichment could enhance seagrass production. Overall, the ecological interaction between *P. oceanica* and amphipods could be seen as a facultative mutualistic relationship. Our results suggest that amphipod mesograzers are key-elements in some of the functional processes regulating these complex and yet endangered ecosystems, which are essential components of Mediterranean coastal zones.

KEY WORDS: Epiphyte grazing, mesograzers, Amphipoda, nutrient cycling, *Posidonia oceanica*

INTRODUCTION

Seagrasses are widespread foundation species, present in many coastal zones throughout the world. They form meadows that constitute key coastal ecosystems, and whose paramount ecological importance is widely recognized (Duarte 2002; Valentine & Duffy 2006). In several (putatively all) meadow ecosystems, the seagrass, the epiphytes that grow on it and the grazers able to consume either the seagrass or its epiphytes are linked by a complex and intricate interplay of reciprocal interactions and feedback loops, termed seagrass/epiphyte/grazer system (Jernakoff et al. 1996). Natural or anthropogenic fluctuations in this system can influence many ecological processes, and ultimately impact the whole meadow functioning (Valentine & Duffy 2006).

The Neptune grass, *Posidonia oceanica* (L.) Delile, is the most widespread seagrass of the Mediterranean Sea. This species is endemic to the Mediterranean and forms large, typically monospecific and fully submerged meadows from shallow depths to 45 meters. The complex tridimensional structure of these meadows offers a suitable habitat to hundreds of animal and plant species, as well as micro-organisms (Buia et al. 2000). In addition, *P. oceanica* supports complex, elaborate food webs (Vizzini 2009). As a result, *P. oceanica* meadows, which

cover up to 50000 km^2 (Bethoux & Copin-Montégut 1986), are biodiversity hotspots in the Mediterranean Sea.

P. oceanica is a large (leaf length up to 150 cm) and long-lived (leaf life span of 9–12 months) seagrass (Gobert et al. 2006). These features allow the development of unique epiphytic communities (*sensu* Borowitzka et al. 2006; i.e. all organisms attached to the exterior surface of the plant). They are one of the most diverse and well-structured communities among all seagrasses, and can represent up to 40% of the foliar biomass (Mazzella et al. 1989). Epiphytes cover all parts of the plant (leaf and rhizomes) and include bacteria, fungi, protozoa, microalgae, macroalgae (mostly crustose and erect Rhodophyta and Phaeophyta), as well as encrusting or erect sessile invertebrates, mainly represented by bryozoans, hydrozoans and polychaetes (Buia et al. 2000). The epiphytic cover is an essential compartment of Neptune grass meadows, and a key feature of *P. oceanica*-associated food webs. Since they have a higher nutritional quality and a better palatability than seagrass leaves or detritus, epiphytes are readily consumed by various animal taxa (Lepoint et al. 2000; Vizzini 2009).

Amphipods (Arthropoda, Malacostraca) are, alongside gastropods and polychaetes, one of the dominant groups of vagile invertebrates found in *P. oceanica* meadows (Gambi et al. 1992). They form an abundant and diverse community, whose dominant taxa graze on epiphytes (Lepoint et al. 2000; Vizzini et al. 2002) with species-specific dietary preferences (Michel et al. in press). Since many fishes rely on them as prey (Bell & Harmelin-Vivien 1983; Pinnegar & Polunin 2000), amphipods constitute an important trophic link to higher trophic levels. However, the ecological significance of these trophic links at the scale of the meadow ecosystem, as well as their functional implications, remain unclear.

In a number of other temperate seagrass systems, amphipod mesograzers (*sensu* Brawley 1992; i.e. organisms whose body size is larger than that of a copepod, but smaller than 2.5 cm) can exert top-down control on epiphytic assemblages (Howard 1982; Neckles et al. 1993; Jernakoff & Nielsen 1997; Duffy & Harvilicz 2001). By doing so, they can release the seagrass from competition for nutrients and/or light, and have positive, indirect effects on seagrass biomass (Duffy et al. 2001; Myers & Heck 2013), production (Neckles et al. 1993), or density (Whalen et al. 2013). Moreover, mesograzers are able, through direct or indirect interactions, to act as regulators and to dampen impacts of environmental changes on meadow ecosystems (e.g. Alsterberg et al. 2013). In *P. oceanica* meadows, gastropods have received some attention (Gacia et al. 2009), but no data exist concerning the influence of epiphyte/amphipod trophic relationships on meadow ecosystem functioning. This limits insights about the actual ecological role of these potentially important mesograzers.

In this context, the objectives of this study were 1) to quantify the impact of amphipod feeding on the epiphytic cover of the leaves of *P. oceanica* and 2) to investigate potential indirect effects of amphipods on their seagrass host. To achieve these goals, we tested the impact of grazer inclusion on biomass of epiphytic functional groups and C/N ratios of *P. oceanica* leaves using in situ microcosms. To account for potential interspecific differences, experiments were focused on three of the dominant species of the community, i.e. *Apherusa chiereghinii* Giordani-Soika, 1949, *Dexamine spiniventris* (Costa, 1853) and *Gammarus aequicauda* (Martynov, 1931). These species display contrasting feeding habits and, taken together, they represent about 60% of the total amphipod abundance in Calvi Bay (Michel 2011; Michel et al. in press).

Neptune grass meadows, like most seagrass ecosystems worldwide, are currently threatened by human activities (Duarte 2002). Through this work, our ultimate goal is to put the trophic relationship between leaf epiphytes and amphipod mesograzers in the wider context of meadow functioning, and therefore to improve

the knowledge of ecological interactions among this remarkably important, yet endangered, ecosystem.

MATERIALS AND METHODS

Experiments were carried out in Calvi Bay (western Mediterranean Sea, north-western Corsica, France). *Posidonia oceanica* meadows cover about 50% of this bay, and reach depths of nearly 40 m. Meadows of Calvi Bay are mostly characterized by a continuous extension, and show important foliar biomass and production (Bay 1984; Gobert et al. 2003). Work was undertaken by scuba diving in the surroundings of the STARESO research station (University of Liège). A circular (radius: 10 m, center coordinates: 42°34'46" N, 8°43'32" E) experimental site was set up in a continuous meadow zone. Depth of the experimental site ranged from 9.5 to 11 m. Meadow density at site depth was 314 ± 121 shoots.m^{-2} (mean ± SD of 45 measurements).

In situ microcosms were set up in this site, directly in the *P. oceanica* meadow. They consisted of 400-µm nylon mesh cylinders (20 cm diameter X 180 cm length). Terminal portions (last 15 cm) of each end were made of elastic fabric, to facilitate microcosm opening, closing and sealing. To place microcosms, a patch of circa 10 *P. oceanica* shoots was randomly selected. Vagile fauna was eliminated by gently shaking the seagrass leaves, in order to cause grazer displacement without destroying the epiphytic cover. Each microcosm was then placed around the leaves. The bottom elastic part was tied around the rhizomes of the shoots, so that amphipods only had access to the foliar stratum. Microcosms were sealed as tight as possible using large plastic cable ties. In addition, each microcosm was anchored to the ground using 2 metal stakes. A float was attached to the top part to ensure adequate position of the microcosm in the water column. Four treatments were considered: one control without grazers, and three others, each containing a single grazer taxon. Each treatment was replicated twice, giving a total of 8 microcosms. In addition, a procedural control consisting of a patch of 10 shoots without microcosm was realized, to ensure that the microcosm itself had no effect on the epiphyte community or the seagrass, notably through shading.

Amphipods were sampled using light traps which were modified after those described by Michel et al. (2010). Each live animal was identified through direct observation and photographs. The accuracy of these identifications was checked at the end of the experiment. All identifications were correct in the cases of *Apherusa chiereghinii* and *Dexamine. spiniventris*. However, a minor proportion (about 5%) of animals considered as being *Gammarus aequicauda* actually belonged to the morphologically close *Gammarus crinicornis* Stock, 1966 or *Gammarus subtypicus* Stock, 1966. Consequently, they will be referred to as "*Gammarus* spp." over the course of this article.

Body size differed across grazer taxa. Specimens of *A. chiereghinii* (total body length 5.48 ± 1.17 mm; mean ± SD) were much smaller than those of *D. spiniventris* (total body length 9.89 ± 1.59 mm; mean ± SD) or *Gammarus* spp. (total body length 12.41 ± 2.59 mm; mean ± SD). To account for these differences, different grazer population sizes were used (50 individuals for *A. chiereghinii*, 20 individuals for *D. spiniventris* and *Gammarus* spp.). These populations respectively correspond to amphipod densities of 707 and 283 ind.m^{-2}, and are within the range commonly encountered in Calvi bay (87–1028 ind.m^{-2}; Sturaro et al. 2015). In all cases, only individuals that could clearly be identified as adults were selected.

Amphipods were added to the corresponding microcosms on 9 June 2009 for one replicate of each treatment, and on 10 June 2009 for the other replicate. During the course of the experiment, maintenance dives were performed twice a week to ensure the metal stakes remained in place, and to gently scrub off the epiphytes that developed

on the microcosm mesh with a soft brush. The experiment ended after 21 days. At this stage, all *P. oceanica* shoots were cut at the rhizome level, and the microcosms were brought back to the laboratory unopened for processing.

Each seagrass shoot (n = 7 to 11, according to the microcosm) was processed separately. *P. oceanica* leaves were checked for grazing marks, and their epiphytes were scraped under a binocular microscope, using a scalpel blade. They were separated into four functional groups according to LEPOINT et al. (2007): erect algae (also referred to as "erect epiflora"), encrusting algae (= "encrusting epiflora"), erect animals (= "erect epifauna") and encrusting animals (= "encrusting epifauna"). Seagrass tissues, epiphytes and grazers were oven-dried at 60°C for 72 h, and their biomass was subsequently determined using an analytical balance (AX105 DeltaRange, Mettler-Toledo, Greifensee, Switzerland). Reproducibility range of successive weighings was ± 0.04 mg.

The basal portions (first 5 cm) of each seagrass leaf blade were cut. All leaf fragments originating from the same shoot were grouped together and ground to a homogeneous powder. Carbon and nitrogen contents of seagrass leaves were determined using a NA1500 elemental analyzer (Carlo Erba, Milano, Italy). Glycine (Merck, Darmstadt, Germany) was used as a standard for elemental contents measures. Analytical precision was 2% of the relative content of samples (i.e. 0.6% for a sample containing 30% of a given element). C/N ratios were calculated using relative organic C and N contents, both expressed in percentage of total dry mass.

Inter-treatment differences of measured parameters were tested using analysis of variance followed by multiple comparison procedures. Since Shapiro-Wilk normality tests revealed that several datasets did not follow a Gaussian distribution, data were log-transformed. Individual shoot measurements were analyzed through nested 1-way ANOVA using "treatment" as a fixed factor and "microcosm" as a random factor nested within treatment. When differences among treatments were present, they were explored using Tukey's HSD post-hoc test. All statistical analyses were conducted using JMP 9.0.0 (SAS Software, Cary, U.S.A.).

RESULTS

Survival rate was low for *Apherusa chiereghinii* (18%; final grazer density 127 ind.m^{-2}), but much higher for *Dexamine spiniventris* (80%; final grazer density 226 ind.m^{-2}). It was 115% in *Gammarus* spp. (final grazer density 325 ind.m^{-2}), suggesting that animals reproduced over the course of the experiment. All microcosms, including control treatments, were contaminated with non-amphipod invertebrates (gastropods or copepod crustaceans), indicating that the defaunation step may not have been sufficient. However, biomass of these undesired animals was always low (less than 5% of amphipod grazer biomass) and was comparable across treatments. It was therefore assumed that their impact was negligible in regard to changes

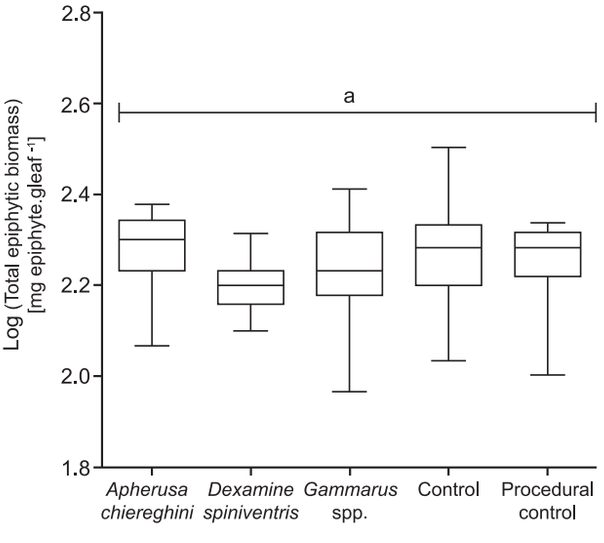

Fig. 1. – Biomass of total epiphytes in each treatment at the end of the grazing experiment, expressed in mg of epiphytes per gram of *Posidonia oceanica* leaf. Central black bars represent medians, box limits are upper and lower quartiles, and error bars represent the full range of the data (minimum-maximum). Different letters indicate statistically different groups (1-way ANOVA & Tukey's HSD post-hoc test, $p < 0.05$).

caused by introduced amphipods. No unplanned amphipod grazers were observed.

At the end of the experiment, the total biomass of epiphytes present on *Posidonia oceanica* leaves (Fig. 1) was similar across treatments (1-way ANOVA, $F_{4,73}$ = 1.70, p = 0.3167), suggesting presence of grazers had no significant effect on the epiphytic community as a whole. However, functional group-specific trends were present (Fig. 2). Grazer presence had no effect on encrusting algae biomass (Fig. 2a; 1-way ANOVA, $F_{4,73}$ = 1.60, p = 0.3489), nor on encrusting animals biomass (Fig. 2b; 1-way ANOVA, $F_{4,73}$ = 0.57, p = 0.6993). On the other hand, biomass of erect algae (Fig. 2c) differed across treatments (1-way ANOVA, $F_{4,73}$ = 41.38, p = 0.0032). It was significantly lower in all grazed treatments than in the "control" and "procedural control" ones (Tukey's HSD post-hoc test, $p < 0.05$ in each case; Fig. 2c). The situation was similar for erect epifauna (Fig. 2d), whose biomass tended to be lower when amphipods were present (1-way ANOVA, $F_{4,73}$ = 64,36, p = 0.0008). As for erect epiflora, this trend was significant for all three grazed treatments (Tukey's HSD post-hoc test, $p < 0.05$ in each case; Fig. 2d).

No seagrass grazing seemed to occur in any of the amphipod-containing microcosms, as no grazing marks or other damage to seagrass

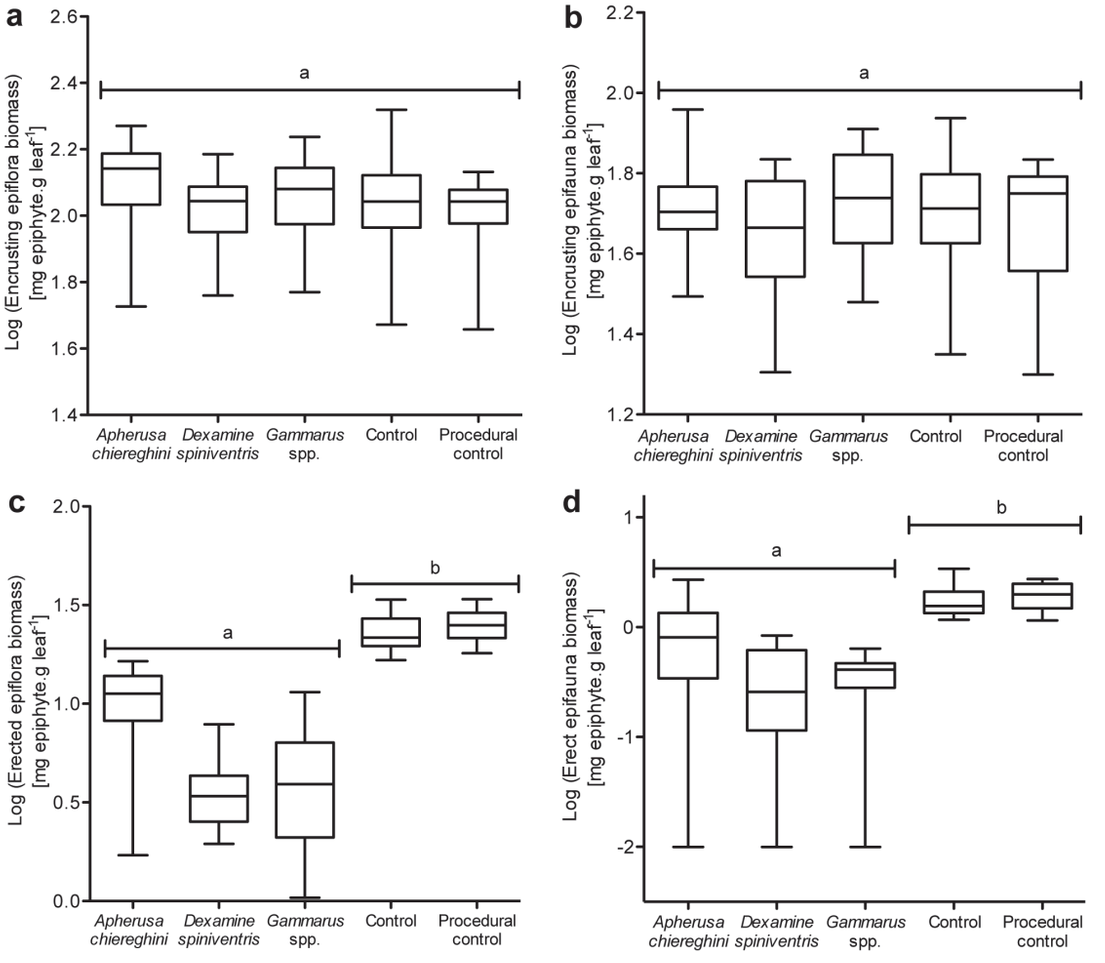

Fig. 2. – Biomass of (a) encrusting algae, (b) encrusting animals, (c) erect algae and (d) erect animals in each treatment at the end of the grazing experiment, expressed in mg of epiphytes per gram of *Posidonia oceanica* leaf. Central black bars represent medians, box limits are upper and lower quartiles, and error bars represent the full range of the data (minimum-maximum). Different letters indicate statistically different groups (1-way ANOVA & Tukey's HSD post-hoc test, $p < 0.05$).

leaves were noted. Grazer presence had an effect on the C/N ratio of *P. oceanica* leaves (1-way ANOVA, $F_{4,73} = 1041.46$, $p < 0.0001$; Fig. 3). It was significantly lower in treatments containing grazers than in both control conditions (control and procedural control; Tukey's HSD post-hoc test, $p < 0.05$ in each case). These lower C/N ratios were linked with higher N content of seagrass leaves, as carbon content was similar in all treatments (data not shown).

No significant effect of the "microcosm [treatment]" factor was detected for any of the performed comparisons (Tukey's HSD post-hoc test, $p > 0.05$ in each case), indicating that none of the analyzed parameters varied across the two microcosms of a single treatment.

DISCUSSION

Amphipods from *Posidonia oceanica* meadows had inconspicuous effects on their host's epiphytic cover. While no effects on total biomass of the epiphytic community, or on the one of crustose morphotypes were seen, the standing stocks of erect epiphytes were lower in the presence of any of the three grazer taxa. This was the case for algae but also for sessile animals. Depletion of epiphytic micro- or macroalgae by amphipods occurs in a number of temperate and subtropical seagrass systems. Experimental discrepancies, alongside differences in biology and life history of amphipods, result in the scattering of amphipod grazing impacts over a broad spectrum (HUGHES et al. 2004). Strong, marked effects are common. In some cases, exclusion of amphipods can cause an increase of over 400% of epiphytic biomass (e.g. CAINE 1980; WHALEN et al. 2013). In this study, impacts were less drastic, as amphipods consumed 50 to 90% of erect algal biomass. This effect is nonetheless more marked than those recorded for other species in different meadows, where amphipods can have moderate and/or low effects on epiphytic abundance (see JASCHINSKI & SOMMER 2008; COOK et al. 2011). Consumption of sessile animals by amphipods, although apparently less generalized, also occurs in other systems. Amphipod grazers from *Zostera marina* meadows feed on erect bryozoans and tunicates, but do not seem to consume the crustose species (DUFFY & HARVILICZ 2001; DOUGLASS et al. 2007). Several of these amphipod taxa can also prey on juvenile bay scallops (*Argopecten irradians*) during their early life stages, when they live on the *Z. marina* blades (LEFCHECK et al. 2014).

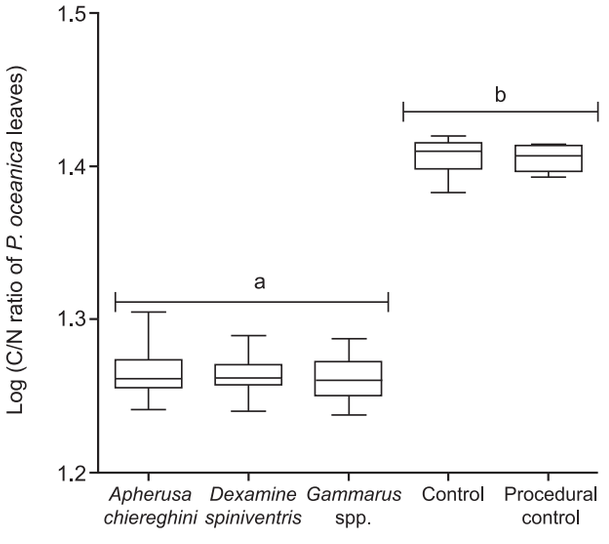

Fig. 3. – C to N ratio of *Posidonia oceanica* leaves in each treatment at the end of the grazing experiment. Central black bars represent medians, box limits are upper and lower quartiles, and error bars represent the full range of the data (minimum-maximum). Different letters indicate statistically different groups (1-way ANOVA & Tukey's HSD post-hoc test, $p < 0.05$).

None of the amphipod grazers seemed to consume encrusting epiphytes. This is consistent with widely observed trends of resistance of crustose algae to herbivory (POORE et al. 2012). Here, it could be linked with the feeding mechanism of the studied amphipods. All three taxa, like most herbivorous amphipods, use the typical feeding mode of gammarid amphipods. It involves cutting fragments through an initial bite from the mandible's incisor process before triturating and crushing them with the mandibular molar process. Food pieces are then gathered and brought to the mouth for ingestion (BELLAN-SANTINI 1999). Crustose morphotypes are not easily accessible to this type of feeding, and amphipods might therefore

simply be unable to consume them. Preferential consumption of erect epiphytes has important implications for the role of amphipod grazers in *P. oceanica* meadows. Their selective grazing pressure may be one of the processes involved in the structuring of the epiphytic cover of seagrass leaves. Discriminatory removal of certain taxa through grazing can indeed relieve the non-consumed species from competition for space, nutrients and/or light, and therefore allow their development and in turn modify the whole epiphytic community structure (JERNAKOFF et al. 1996; JASCHINSKI et al. 2010). On *P. oceanica* leaves, epiphytic biomass is at its lowest in winter. Organisms start to grow during spring. The fast-growing erect brown algae typically dominate the community in spring and early summer (May/June). Crustose epiphytes, such as red coralline algae, are present all year round, but become more and more abundant as the epiphytic cover develops. They are the dominant organisms in late summer, when epiphytic coverage and specific diversity are maximal (MAZZELLA et al. 1989; CEBRIAN et al. 1999; LEPOINT et al. 2000). Amphipods could play a part in this process. By grazing on erect algae, they could limit their biomass, and indirectly favor growth of crustose algae. In doing so, they would participate in the balance between the two epiphytic morphotypes, and allow the epiphytic community to fully develop, and reach its maximal diversity.

Amphipods are not the only mesograzers to impact epiphytic communities in Neptune grass meadows. Gastropods can indeed consume 54 to 70% of the total epiphytic biomass present on *P. oceanica* leaves (GACIA et al. 2009). Moreover, in *P. oceanica* meadows, the studied amphipods only consume macroepiphytes (MICHEL et al. in press) and only feed on erect morphotypes, while gastropods can use their radula to scrape the surface of the leaves and consume microepiphytes (mostly diatoms and bacteria; PEDUZZI 1987; MAZZELLA & RUSSO 1989; GACIA et al. 2009) and, to a lesser extent, crustose macroepiphytes (MAZZELLA & RUSSO 1989). The complementarity of feeding modes could lead to synergetic effects of these two grazer taxa on the epiphytic communities, as biodiversity of grazer assemblages can, through horizontal interactions, modulate their influence on other compartments of the ecosystem. (DUFFY et al. 2001; DUFFY et al. 2003).

C/N ratios of basal portions of *P. oceanica* leaves were significantly lower in all grazed treatments. This was caused by a generalized trend towards N enrichment of growing host tissues when grazers were present. This enrichment could simply be an indirect effect of epiphyte consumption. Since epiphytic biomass decreases through grazing, nitrogen availability would be higher for the surviving organisms, leading to an apparent concentration effect. However, since leaf biomass exceeds by far erect macroalgae biomass, it is more likely that other, non-exclusive phenomena occur concomitantly. Grazing activity itself may directly enhance N cycling by processes such as excretion (fecal pellets and NH_4^+) and/or sloppy feeding. Excretion of either sessile (e.g. bryozoans; HURD et al. 1994) or vagile (BRACKEN et al. 2007) invertebrates can cause N enrichment in tissues of host seaweeds. In *Zostera marina* meadows, slow-moving gastropods can enhance N content of primary producers, while amphipod and isopod mesograzers fail to do so (JASCHINSKI & SOMMER 2010). This suggests that enrichment could only occur in the case of a tight association with seagrass leaves, and that dispersal and dilution of waste products would limit the fertilization effect in the case of highly motile and free-swimming crustaceans (JASCHINSKI & SOMMER 2010). Our results disagree with this hypothesis. The widely different general N availability in the two systems probably explains most of this difference. The Mediterranean Sea in general, and Calvi Bay in particular, are oligotrophic areas (LEPOINT et al. 2004), where plant growth can be limited by nutrient scarcity. Increase of nutrient supply through grazing could be more crucial there than in *Z. marina* meadows of the Baltic, and therefore cause stronger and more marked effects.

Nutrient additions have contrasting impacts on seagrass production (HUGHES et al. 2004). Since epiphytes are often able to use these nutrients more efficiently (higher uptake and growth rates) than the seagrass itself (LEPOINT et al. 2007), they tend to outgrow the seagrass, and can lead to seagrass death in some situations (BOROWITZKA et al. 2006). However, under top-down control of epiphytic growth by mesograzers, this effect is suppressed, and enhanced nutrient availability can have positive effect on seagrass production (HAYS 2005). Growth of *P. oceanica* can be enhanced by in situ nutrient fertilization (ALCOVERRO et al. 1997). In Calvi Bay meadows, low nutrient availability and constant grazing of fast-growing erect epiphytes by amphipods suggest that N enrichment could have a positive effect on seagrass growth.

Contrary to other grazer groups, crustaceans globally benefit seagrasses (POORE et al. 2012). However, the interaction between crustaceans and seagrasses can turn antagonistic. Some taxa (idoteid isopods, ampithoid amphipods) graze directly on seagrass tissues when alternative food supplies are low (VALENTINE & DUFFY 2006). During our experiment, no grazing marks were observed. Moreover, under natural conditions, none of the dominant amphipods of *P. oceanica* meadows feed on their seagrass host (MICHEL et al. in press). The interaction has therefore no reason to become negative. Instead, amphipod mesograzers have two indirect, putatively positive effects on their seagrass host's production. First, through their feeding activity, they may release Neptune grass from competition for nutrients and/or light with faster-growing erect epiphytes. Second, through excretion and/or sloppy feeding, they may enhance nutrient cycling, and in turn boost seagrass production. The ecological interaction between *P. oceanica* and amphipod grazers could therefore be seen as a facultative mutualistic relationship, where amphipods would keep biomasses of fast-growing erect algal competitors at acceptable levels and supply nutrient for host growth, while the seagrass would provide trophic resources for amphipods, as well as a substratum and a shelter from predation (VALENTINE & DUFFY 2006).

Functional interactions among the seagrass/epiphyte/grazer system form a complex and entangled network, where multiple factors can directly or indirectly influence plant and animal components (JERNAKOFF et al. 1996). Unraveling the elaborate interactions between Neptune grass, epiphytes growing on its leaves and mesograzers inhabiting its meadows is a complicated task, and requires further work on many aspects. This study nevertheless presented results that constitute, to the best of our knowledge, the first direct, experimental evidence of the importance of amphipod grazers in trophofunctional relationships among *Posidonia oceanica* meadows. For this reason, it provides another step towards a better comprehension of this complex, pivotal, yet critically endangered, ecosystem.

ACKNOWLEDGEMENTS

Loïc Michel was a Belgian Funds for Scientific Research (F.R.S-FNRS) research fellow (grant number FC74034). Gilles Lepoint is a F.R.S.-FNRS research associate. The authors would like to thank Renzo Biondo (Laboratory of Oceanology, ULg) for his technical assistance in the conception and building of sampling and experimental gear, the staff of the STARESO Research station for their help during the fieldwork, and Joseph G. Schnitzler (Laboratory of Oceanology, ULg) for his assistance with statistical analyses. Constructive comments from Dorothée Pête (Laboratory of Oceanology, ULg) and from two anonymous reviewers helped to improve earlier versions of this article. This paper is MARE publication nr. 291.

REFERENCES

ALCOVERRO T, ROMERO J, DUARTE CM & LOPEZ NI (1997). Spatial and temporal variations in nutrient limitation of seagrass *Posidonia oceanica* growth in the NW Mediterranean. Marine Ecology Progress Series, 146: 155-161.

ALSTERBERG C, EKLOF JS, GAMFELDT L, HAVENHAND JN & SUNDBACK K (2013). Consumers mediate

the effects of experimental ocean acidification and warming on primary producers. Proceedings of the National Academy of Sciences of the United States of America, 110(21): 8603-8608.

Bay D (1984). A field study of the growth dynamics and productivity of *Posidonia oceanica* (L.) Delile in Calvi Bay, Corsica. Aquatic Botany, 20(1-2): 43-64.

Bell JD & Harmelin-Vivien ML (1983). Fish fauna of French Mediterranean *Posidonia oceanica* seagrass meadows. 2. Feeding habits. Tethys, 11: 1-14.

Bellan-Santini D (1999) Ordre des Amphipodes (Amphipoda Latreille, 1816). In: Forest J (ed), Traité de Zoologie - Anatomie, Systématique, Biologie (Pierre-P. Grassé). Tome VII, Fascicule III A : Crustacés Péracarides. Institut Océanographique de Monaco, Monaco: 93-176.

Bethoux J & Copin-Montégut G (1986). Biological fixation of atmospheric nitrogen in the Mediterranean Sea. Limnology and Oceanography, 31(6): 1353-1358.

Borowitzka MA, Lavery P & van Keulen M (2006) Epiphytes of seagrasses. In: Larkum AWD, Orth RJ & Duarte CM (eds), Seagrasses : Biology, Ecology and Conservation. Springer: 441-461.

Bracken MES, Gonzalez-Dorantes CA & Stachowicz JJ (2007). Whole-community mutualism: associated invertebrates facilitate a dominant habitat-forming seaweed. Ecology, 88(9): 2211-2219.

Brawley HS (1992) Mesoherbivores. In: John DM, Hawkins SJ & Price JH (eds), Plant-Animal Interactions in the Marine Benthos. Clarendon Press, Oxford: 235-263.

Buia MC, Gambi MC & Zupo V (2000). Structure and functioning of Mediterranean seagrass ecosystems: an overview. Biologia Marina Mediterranea, 7: 167-190.

Caine EA (1980). Ecology of two littoral species of caprellid amphipods (Crustacea) from Washington, USA. Marine Biology, 56(4): 327-335.

Cebrian J, Enriquez S, Fortes M, Agawin N, Vermaat JE & Duarte CM (1999). Epiphyte accrual on *Posidonia oceanica* (L.) Delile leaves: implications for light absorption. Botanica Marina, 42: 123-128.

Cook K, Vanderklift MA & Poore AGB (2011). Strong effects of herbivorous amphipods on epiphyte biomass in a temperate seagrass meadow. Marine Ecology Progress Series, 442: 263-269.

Douglass JG, Duffy JE, Spivak C & Richardson JP (2007). Nutrient versus consumer control of community structure in a Chesapeake Bay eelgrass habitat. Marine Ecology Progress Series, 348: 71-83.

Duarte CM (2002). The future of seagrass meadows. Environmental Conservation, 29(2): 192-206.

Duffy JE & Harvilicz AM (2001). Species-specific impacts of grazing amphipods in an eelgrass-bed community. Marine Ecology Progress Series, 223: 201-211.

Duffy JE, Richardson JP & Canuel EA (2003). Grazer diversity effects on ecosystem functioning in seagrass beds. Ecology Letters, 6: 637-645.

Duffy JE, MacDonald KS, Rhode JM & Parker JD (2001). Grazer diversity, functional redundancy, and productivity in seagrass beds: an experimental test. Ecology, 82(9): 2417-2434.

Gacia E, Costalago D, Prado P, Piorno D & Tomas F (2009). Mesograzers in *Posidonia oceanica* meadows: an update of data on gastropod-epiphyte-seagrass interactions. Botanica Marina, 52(5).

Gambi MC, Lorenti M, Russo GF, Scipione MB & Zupo V (1992). Depth and seasonal distribution of some groups of the vagile fauna of the *Posidonia oceanica* leaf stratum: Structural and trophic analyses. Marine Ecology, 13(1): 17-39.

Gobert S, Kyramarios M, Lepoint G, Pergent-Martini C & Bouquegneau JM (2003). Variations at different spatial scales of *Posidonia oceanica* (L.) Delile beds; effects on the physico-chemical parameters of the sediment. Oceanologica Acta, 26(2): 199-207.

Gobert S, Cambridge ML, Velimirov B, Pergent G, Lepoint G, Bouquegneau JM, Dauby P, Pergent-Martini C & Walker DI (2006) Biology of *Posidonia*. In: Larkum AWD, Orth RJ & Duarte CM (eds), Seagrasses : Biology, Ecology and Conservation: 387-408.

Hays CG (2005). Effect of nutrient availability, grazer assemblage and seagrass source population on the interaction between *Thalassia testudinum* (turtle grass) and its algal epiphytes. Journal of Experimental Marine Biology and Ecology, 314(1): 53-68.

Howard RK (1982). Impact of feeding activities of epibenthic amphipods on surface-fouling of eelgrass blades. Aquatic Botany, 14: 91-97.

Hughes AR, Bando KJ, Rodriguez LF & Williams SL (2004). Relative effects of grazers and nutrients on seagrasses: a meta-analysis approach. Marine Ecology Progress Series, 282: 87-99.

Hurd CL, Durante KM, Chia FS & Harrison PJ (1994). Effect of bryozoan colonization on inorganic nitrogen acquisition by the kelps *Agarum fimbriatum* and *Macrocystis integrifolia*. Marine Biology, 121(1): 167-173.

Jaschinski S & Sommer U (2008). Functional diversity of mesograzers in an eelgrass–epiphyte system. Marine Biology, 154(3): 475-482.

Jaschinski S & Sommer U (2010). Positive effects of mesograzers on epiphytes in an eelgrass system. Marine Ecology Progress Series, 401: 77-85.

Jaschinski S, Flöder S & Sommer U (2010). Consumer identity, abundance and nutrient concentration affect epiphyte diversity in an experimental eelgrass system. Oikos, 119(11): 1745-1754.

Jernakoff P & Nielsen J (1997). The relative importance of amphipod and gastropod grazers in *Posidonia sinuosa* meadows. Aquatic Botany, 56(3-4): 183-202.

Jernakoff P, Brearley A & Nielsen J (1996). Factors affecting grazer-epiphytes interactions in temperate seagrass meadows. Oceanography and Marine Biology: an Annual Review, 34: 109-162.

Lefcheck JS, van Montfrans J, Orth RJ, Schmitt EL, Duffy JE & Luckenbach MW (2014). Epifaunal invertebrates as predators of juvenile bay scallops (*Argopecten irradians*). Journal of Experimental Marine Biology and Ecology, 454: 18-25.

Lepoint G, Gobert S, Dauby P & Bouquegneau JM (2004). Contributions of benthic and planktonic primary producers to nitrate and ammonium uptake fluxes in an nutrient-poor shallow coastal area (Corsica, NW Mediterranean). Journal of Experimental Marine Biology and Ecology, 302: 107-122.

Lepoint G, Nyssen F, Gobert S, Dauby P & Bouquegneau J-M (2000). Relative impact of a seagrass bed and its adjacent epilithic algal community in consumer diets. Marine Biology, 136: 513-518.

Lepoint G, Jacquemart J, Bouquegneau JM, Demoulin V & Gobert S (2007). Field measurements of inorganic nitrogen uptake by epiflora components of the seagrass *Posidonia oceanica* (Monocotyledons, Posidoniaceae). Journal of Phycology, 4(2): 208-218.

Mazzella L & Russo GF (1989). Grazing effect of two *Gibbula* species (Mollusca, Archaeogastropoda) on the epiphytic community of *Posidonia oceanica* leaves. Aquatic Botany, 35: 353-373.

Mazzella L, Scipione MB & Buia MC (1989). Spatio-temporal distribution of algal and animal communities in a *Posidonia oceanica* meadow. Marine Ecology, 10(2): 107-129.

Michel L (2011). Multidisciplinary study of trophic diversity and functional role of amphipod crustaceans associated to *Posidonia oceanica* meadows. PhD in Sciences thesis, University of Liège, Belgium.

Michel L, Lepoint G, Dauby P & Sturaro N (2010). Sampling methods for amphipods of *Posidonia oceanica* meadows: a comparative study. Crustaceana, 83(1): 39-47.

Michel LN, Dauby P, Gobert S, Graeve M, Nyssen F, Thelen N & Lepoint G (in press). Dominant amphipods of *Posidonia oceanica* seagrass meadows display considerable trophic diversity. Marine Ecology.

Myers JA & Heck KLJ (2013). Amphipod control of epiphyte load and the concomitant effects on shoalgrass *Halodule wrightii* biomass. Marine Ecology Progress Series, 483: 133-142.

Neckles HA, Wetzel RL & Orth RJ (1993). Relative effects of nutrient enrichment and grazing on epiphyte-macrophyte (*Zostera marina* L.) dynamics. Oecologia, 93: 285-295.

Peduzzi P (1987). Dietary preferences and carbon absorption by two marine gastropods, *Gibbula umbilicaris* (Linné) and *Jujubinus striatus* (Linné). Marine Ecology, 8(4): 359-370.

Pinnegar JK & Polunin NVC (2000). Contributions of stable-isotope data to elucidating food webs of Mediterranean rocky littoral fishes. Oecologia, 122(3): 399-409.

Poore AG, Campbell AH, Coleman RA, Edgar GJ, Jormalainen V, Reynolds PL, Sotka EE, Stachowicz JJ, Taylor RB, Vanderklift MA & Duffy JE (2012). Global patterns in the impact of marine herbivores on benthic primary producers. Ecological Letters, 15(8): 912-922.

Sturaro N, Lepoint G, Vermeulen S & Gobert S (2015). Multiscale variability of amphipod assemblages in *Posidonia oceanica* meadows. Journal of Sea Research, 95: 258-271.

Valentine JF & Duffy JE (2006) The central role of grazing in seagrass ecology. In: Larkum AWD, Orth RJ & Duarte CM (eds), Seagrasses : Biology, Ecology and Conservation. Springer: 463-501.

Vizzini S (2009). Analysis of the trophic role of Mediterranean seagrasses in marine coastal ecosystems: a review. Botanica Marina, 52(5): 383-393.

Vizzini S, Sarà G, Michener RH & Mazzola A (2002). The role and contribution of the seagrass *Posidonia oceanica* (L.) Delile organic matter for secondary consumers as revealed by carbon and nitrogen stable isotope analysis. Acta Oecologica, 23: 277-285.

Whalen MA, Duffy JE & Grace JB (2013). Temporal shifts in top-down vs. bottom-up control of epiphytic algae in a seagrass ecosystem. Ecology, 94(2): 510-520.

Received: October 10th, 2014

Accepted: March 19th, 2015

Branch editor: Isa Schön

Belg. J. Zool., 145(2) : 95-102 July 2015

Habitat comparison of *Mideopsis orbicularis* (O. F. Müller, 1776) and *M. crassipes* Soar, 1904 (Acari: Hydrachnidia) in the Krąpiel River

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ABSTRACT. Ecological studies of water mites have a very long tradition. However, no explicit data have been obtained to date with regard to specific ecological parameters defining autoecological values for particular species, and therefore such values have not been compared between closely related species. The present study is an attempt at making such comparisons between two closely related species: *Mideopsis orbicularis* and *Mideopsis crassipes*. Both species are psammophilous; *M. orbicularis* prefers stagnant waters, while *M. crassipes* prefers running waters. The research was conducted during 2010 in 89 localities distributed along the Krąpiel River and in water reservoirs found in its valley. The two species were collected solely in the river, where they were found in 26 localities and only these localities were analyzed. Until now *M. crassipes* was characterized as a species preferring rather fast-flowing habitats, and *M. orbicularis* as preferring slow water habitats, i.e. isolated still-water bodies. In this study both species preferred slow flow water habitats: 77.5% (225 individuals) of all *M. orbicularis* specimens and 67.3% (318 individuals) of all *M. crassipes* specimens were collected in isolated still-water bodies. The only correlations identified between water mite occurrence and water quality were the positive one between the abundance of *M. orbicularis* and water temperature, the negative one between the abundance of this species and BOD_5. There were also some correlations with substrate, including the positive correlation between occurrence of *M. crassipes* and sandy bottom. *M. orbicularis* was also encountered on organic bottoms and among water plants.

KEY WORDS: water mites, bottom, BOD_5, oxygen, temperature

INTRODUCTION

Studies of water mite ecology have a long tradition and thus the ecological characteristics of most species have already been established. A comparatively large number of publications have been devoted to the association between vertical oxygen distribution and the presence of water mites within a lake basin (Viets, 1930, 1931; Pieczyński, 1959; Kowalik 1973, 1977, 1978, 1984; Meyer & Schwoerbel, 1981; Zawal, 2007; Zawal & Stępień, 2007). Cichocka's (1998) study showed correlations between hydrochemical parameters and the occurrence of water mites in peat bogs, while works of several other authors (Cicolani & Di Sabatino, 1991; Gerecke & Schwoerbel, 1991; Di Sabatino et al., 2000; Stur et al., 2005; Camacho et al., 2006; Van Haaren & Tempelman, 2009; Martin et al. 2010; Bottazzi et al., 2011; Stoch et al., 2011) investigated the connection between the presence of water mites and other

invertebrates, and physico-chemical parameters of lotic waters. The present paper compares the habitat occurrence of two closely related species, *Mideopsis orbicularis* and *Mideopsis crassipes*, inhabiting the valley of a rather small lowland river: the Krąpiel. According to data from literature (Viets, 1936; Biesiadka & Kowalik, 1979; Gerecke, 2002), both of these species show preference for sandy bottoms, but the first of them inhabits mainly lentic waters, while the latter prefers lotic waters. However, under certain conditions the species co-occur in the same habitats. This refers mainly to small and medium-sized lowland rivers. The Krąpiel River, where studies on macrobenthos distribution, water mite fertility and the impact of river dredging on the fauna of invertebrates and vegetation have been conducted (Keszka & Raczyński, 2004; Raczyńska & Machula, 2006; Zawal, 2009; Dierzgowska & Zawal, 2010; Buczyński et al., 2011; Kłosowska et al., 2011; Kurzątkowska & Zawal, 2011; Szlauer-Łukaszewska & Zawal, 2013, Stępień et al., 2015, Zawal et al., 2015) was an excellent site for checking patterns of occurrence of the two species in various habitats in relationship to physico-chemical parameters of water and the bottom structure. It was hypothesized that main parameters affecting the occurrence of the two species include flow velocity, sediment type, degree of vegetation coverage of the bottom and oxygen content. It was assumed that *M. crassipes* would occur in habitats characterized by a more rapid water flow and higher oxygen content.

MATERIALS AND METHODS

The study was based on material collected for the purpose of a project examining the effect of landscape structure on the distribution of selected groups of aquatic invertebrates in a small lowland river. Fieldwork was conducted from May until October, 2010. The research covered the whole length of the river where 89 research sites were established in 13 locations (Fig. 1), distributed in such a way as to cover all habitat types in which water mites occurred. Samples were collected from both lotic and lentic waters with a triangular hand net. Each sampling consisted of 10 energetic sweeps and covered an area of ca. 0.5 m^2. Three subsamples were collected from each site for the purpose of variability analyses. Further analysis focused on those sites where at least one of the two mite species was encountered at least once. In total, 546 samples were collected from 26 sites situated solely in lotic waters.

The water parameters: temperature, pH, electrolytic conductivity and dissolved oxygen content were measured with an Elmetron CX-

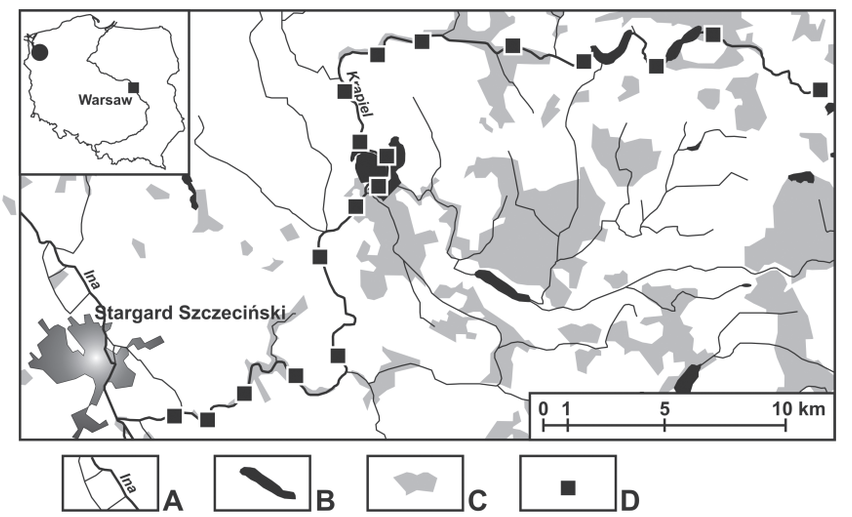

Fig. 1. – Location of the research sites: A – rivers, B – lakes and fish ponds, C – forests, D – research sites.

Table 1

Correlations between number of specimens and water parameters.

Parameters	Spearman's correlations. In **bold** significance; p <0.05											
	O_2	pH	Temp.	Cond.	NH_4	NO_3	PO_3	Fe	Turb.	Hard	BOD_5	Curr
M. orbicularis	0,089	0,076	**0,224**	-0,143	-0,034	0,092	-0,023	0,104	-0,071	0,082	**-0,34**	-0,069
M. crassipes	0,156	0,123	0,074	0,175	-0,045	0,059	-0,096	0,057	0,12	0,103	0,067	0,137

401 multiparametric sampling probe; water flow using a SonTek acoustic FlowTracker flowmeter; BOD_5 by Winkler`s method, and NH_4, NO_3, PO_3, Fe, turbidity, hardness with the help of Slandi LF205 photometer. Three measurements were performed every time and the median was used for further analyses. The following statistical methods were used for data analysis: the chi-squared test – to identify differences in the sex ratio and preferences regarding bottom granularity; Spearman's correlation to identify the correlation between the abundance of species and physico-chemical parameters of water; discrimination analysis and Mann-Whitney U test to identify the correlation between species distribution and physico-chemical parameters of water; and the non-parametric ANOVA Kruskal-Wallis test to identify seasonal changes in a number of specimens. All analyzes were performed using Statistica 9.0 PL.

RESULTS

Water mites representing the genus *Mideopsis* were found in 26 of the 89 sites sampled; the presence of *M. orbicularis* was recorded in 23 sites, the presence of *M. crassipes* in 24 sites, and 22 sites were inhabited by both species simultaneously. All sites were associated with the river bed (Fig. 1). The sites situated in the river current were inhabited by two species much more frequently (18 sites) and at higher abundance than those situated in isolated still-water bodies (8 sites) the differences were not statistically significant. In total, 762 specimens were of mites collected: 290 individuals of *M. orbicularis* and 472 individuals of *M. crassipes*. Statistically significant correlations were found between abundance *M. orbicularis* and temperature (positive correlation) and BOD_5 (negative correlation) (Table 1).

BOD_5 was the only parameter with discriminative value among all hydrochemical factors considered (Wilks' Lambda distribution: 0.91670; approximate F-distribution: (1.80) = 7.269; $p < 0.008$) and displayed a statistically significant difference between the species (Mann-Whitney's U test: $Z = -2.246$; $p = 0.025$), revealing a much higher tolerance in the case of *M. crassipes*.

M. crassipes displayed a significant positive relationship with a mineral bottom (Mann-Whitney's U test: $Z = 2.635$; $p = 0.008$) and was more common in habitats without plants ($Z = -2.145$; $p = 0.031$). Chi square tests revealed statistically significant differences in relation to the structure of the bottom where each species occurred ($Chi^2 = 228{,}239$ df = 8 $p < 0.0001$). Furthermore, *M. crassipes* appeared to prefer bottoms characterized by larger grain sizes than *M. orbicularis* (Fig. 2).

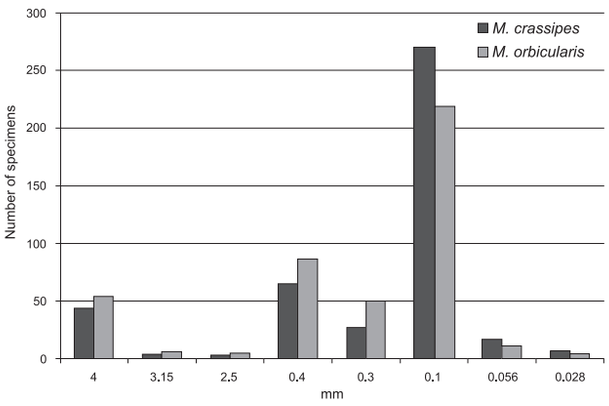

Fig. 2. – The occurrence of the species depending of the size of the ground grain.

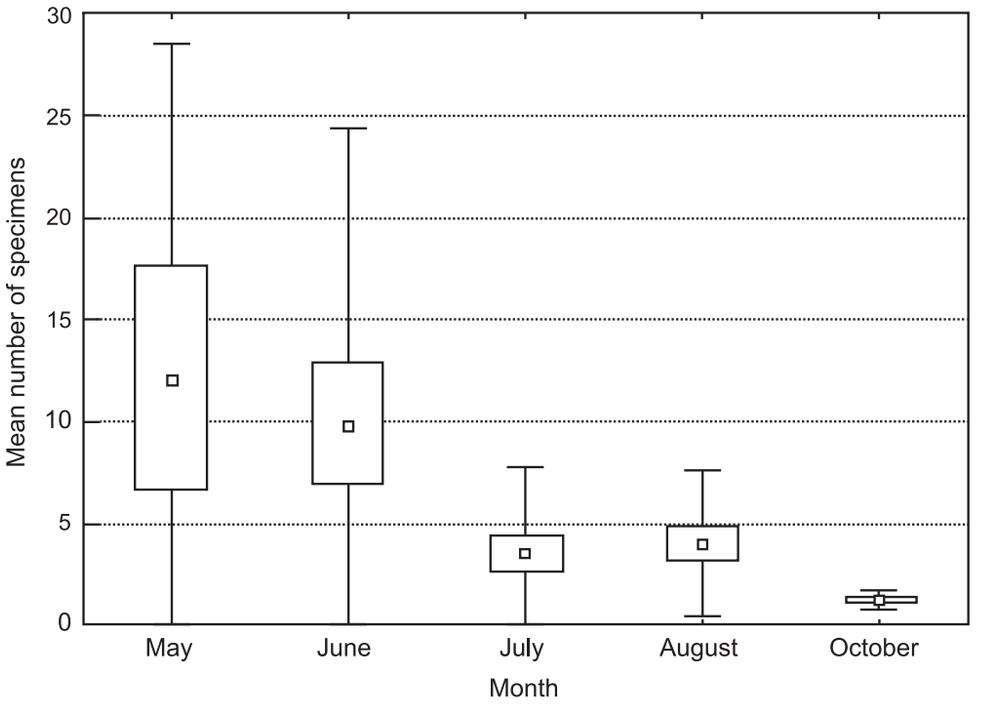

Fig. 3. – Changes of number of specimens of *M. orbicularis* during the whole research period.

Both species showed largest numbers of individuals present in early summer and a decline through to autumn (Figs 3-4). Results of the Kruskal-Wallis test showed that those changes were statistically significant for *M. crassipes* (H (4, N = 84) = 11.497 p = .0215)), but not for *M. orbicularis* (H (4, N = 84) = 7.759 p = 0.101)). In the case of *M. orbicularis* the lack of significance may be associated with high type II error (low N, the power of the test).

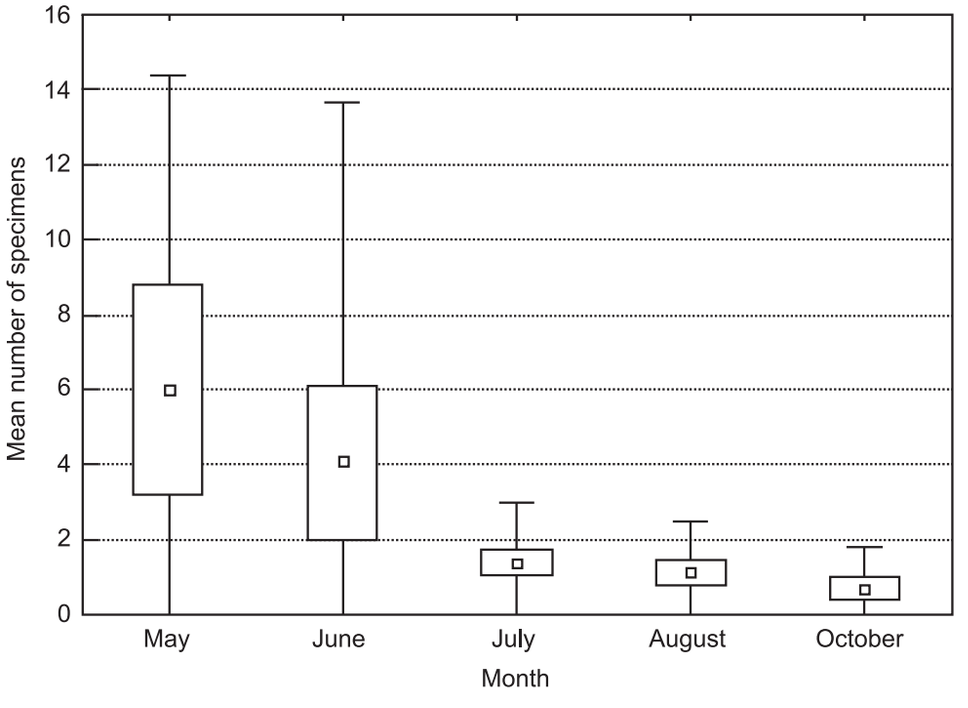

Fig. 4. – Changes of number of specimens of *M. crassipes* during the whole research period.

DISCUSSION

Both *M. orbicularis* and *M. crassipes* show preference for sandy bottoms (Viets, 1936; Biesiadka & Kowalik, 1979; Martin, 1997) and both of them prey on the Cladocera and larvae of the Diptera, and parasitise the Chironomidae (Martin, 2008). So far, the first species has been found mostly in lentic waters (Biesiadka, 1972; Kowalik, 1984; Bagge & Meriläinen, 1985; Zawal 1992; Cichocka, 1998), although it has also occasionally been recorded in lotic waters (Cichocka, 1996; Stryjecki, 2002; Biesiadka et al., 2004; Zawal 2006). As for the latter species, it is a typically rheophilous one (Cichocka, 1996; Stryjecki, 2002; Biesiadka et al., 2004). In the area studied, both species occurred exclusively in river habitats, avoiding lentic water bodies in the river valley. Similar results were obtained by Biesiadka et al. (2004). This is due to the character of the valley water bodies, which are very eutrophic and overgrown, and have bottoms covered with a thick layer of mud. The analysis of data from literature (Cichocka, 1996; Stryjecki, 2002; Biesiadka et al., 2004; Zawal 2006), and results of the present study, show that *M. crassipes* is a typically rheophilous species, preferring rather fast-flowing rivers, while *M. orbicularis* is distributed over two habitat types: slow-flowing rivers and lentic waters, with a tendency to prefer the latter. The reason more flowing sites were occupied by the two species in comparison to sites in isolated still-water bodies was certainly due to the fact that the latter sites were definitely less numerous and preferences of the species. *M. crassipes* occupied the two habitats in approximately equal abundance while *M. orbicularis* was more abundant in isolated still-water bodies.

It is interesting to observe an almost total lack of correlation between the investigated physico-chemical parameters of water and the abundance of the studied species. Such correlations have been identified for some water mite species and other invertebrates inhabiting lotic waters (Cicolani & Di Sabatino, 1991; Gerecke & Schwoerbel, 1991; Di Sabatino et al., 2000; Camacho et al., 2006; Bottazzi et al., 2011) and most frequently were connected with low temperature, high oxygen content and water pH (Kowalik, 1978, 1984; Cichocka, 1998; Zawal, 2007; Zawal & Stępień, 2007). The only correlations identified in our study were the positive one between the abundance of *M. orbicularis* and water temperature and the negative one between the abundance of this species and BOD_5. This correlation confirmed the more eurythermic character of *M. orbicularis*, reflecting its occurrence in standing waters. The effect of other parameters on its occurrence was probably limited to an indirect effect on *M. orbicularis* through influencing the amount of oxygen. As water turbulence in the river guarantees a constant supply of oxygen, the remaining physico-chemical parameters of the water can be considered to have a negligible effect on the oxygen content in the water. This, of course, applies to rivers that are relatively clean. In polluted rivers decomposition processes consume oxygen, leading to a reduction in the number of water mite species. (Cicolani & Di Sabatino, 1991; Gerecke & Schwoerbel, 1991).

It is believed that both species are associated with a sandy bottom, but our data clearly confirmed this correlation only in the case of *M. crassipes*. *M. orbicularis* was also encountered in the sites with organic bottoms and among water plants. According to data from previous studies (Biesiadka, 1972; Kowalik, 1984; Bagge & Meriläinen, 1985; Zawal, 1992; Cichocka, 1994), *M. orbicularis* inhabiting lakes prefers sandy bottoms, but in rivers it also inhabits sites where organics are present and sites that are overgrown with plants (Cichocka, 1996; Biesiadka et al., 2004). As for *M. crassipes*, it has been encountered almost solely over mineral bottoms, whether it was a sandy bottom or one covered with sand and pebbles, and sometimes also on bottoms covered by the plant periphyton (Cichocka, 1996; Biesiadka et al., 2004).

There appeared to be some differences between the two species in the study area in terms of the grain sizes of the bottom, albeit the species co-occurred at most sites. *M. crassipes* was associated with more fine-grained bottoms than *M. orbicularis*. This contrasts with previous classification of *M. orbicularis* as a typically psammophilous species (Cichocka, 1996; Biesiadka et al., 2004). Gerecke (2002) and suggests that *M. orbicularis* is a lenitobiont species, and its presence in rivers is associated with the presence of detritus in the substrate. The current research showed that both species preferred the mineral substrate, although the habitats where *M. orbicularis* dominated were characterized by a slightly higher detritus content. It seems that the psammophilous character of *M. orbicularis* is clearly stronger in stagnant water, which is probably associated with a higher amount of oxygen present on the substrate. However, in the lotic waters this species has a slightly wider range of occurrence and may also occur on gravelly bottoms.

Summing up the above characteristics, it may be stated that *M. crassipes* is a species much more closely associated with lotic water habitats than *M. orbicularis* and in rivers it prefers habitats that are closer to the river current.

ACKNOWLEDGEMENTS

Financial support was provided by the Ministry of Science and Higher Education no. N305 222537.

REFERENCES

Bagge P & Meriläinen JJ (1985). The occurrence of water mites (Acari: Hydrachnellae) in the estuary of the River Kyrönjoki (Bothnian Bay). Ann. Zool. Fenn., 22: 123-127.

Biesiadka E (1972). Wodopójki (Hydracarina) Wielkopolskiego Parku Narodowego. Prace monogr. Przyr. Wielkop. Parku Nar., 5: 1-103.

Biesiadka E & Kowalik W (1979). A new species of *Mideopsis* Neuman (Hydrachnellae, Acari) from Poland. Bull. Acad. Pol. Sci., Cl. II, 26(10): 695-702.

Biesiadka E, Cichocka M & Moroz MD (2004). Water mites (Hydrachnidia) from the Neman River (Belarus), some of its tributaries and riverine reservoirs. Fragmenta faunistica, 47: 143-164.

Buczyński P, Przewoźny M, Zawal A & Zgierska M (2011). On the occurrence of *Potamophilus acuminatus* (Fabricius, 1772) (Coleoptera: Elmidae) in Poland. Baltic J. Coleopterol., 11: 45-56.

Bottazzi E, Bruno MC, Pieri V, Di Sabatino A, Silveri L, Carolli M & Rossetti G (2011). Spatial and seasonal distribution of invertebrates in Northern Apennine rheocrene springs. In: Cantonati M, Gerecke R, Jüttner I & Cox EJ (eds), Springs: neglected key habitats for biodiversity conservation: 77-92. J. Limnol., 70 (Suppl. 1), http://dx.doi.org/10.3274/JL11-70-S1-05.

Camacho AI, Valdecasas AG, Rodríguez J, Cuezva S, Lario J & Sánchez-Moral S (2006). Habitat constraints in epikarstic waters of the Iberian Peninsula cave system. Ann. Limnol. - Int. J. Lim. 42(2): 127-140. http://dx.doi.org/10.1051/limn/2006009

Cichocka M (1996). Wodopójki (Hydracarina) rzeki Pasłęki. Fragm. Faun., 39(14): 179-205.

Cichocka M (1998). Wodopójki (Hydracarina) torfowisk Pojezierza Mazurskiego. Studium faunistyczno-ekologiczne. Studies and materials of WSP Olsztyn, Olsztyn.

Cicolani B & Di Sabatino A (1991). Sensitivity of water mites to water pollution. In: Dusbàbek F & Bukva V (eds), Modern Acarology. Akademia, Prague and SPB Academic Publishing, The Hague, Vol. 1: 465-475

Di Sabatino A, Gerecke R & Martin P (2000). The biology and ecology of lotic water mites (Hydrachnidia). Freshwater Biol. 44: 47-62.

Dzierzgowska K & Zawal A (2010). *Hydrodroma pilosa* BESSELING, 1940 and *Limnesia undulatoides* DAVIDS, 1997 (ACARI, HYDRACHNIDIA) – New records from Poland. Natura Montenegrina, 9 (3): 451-455.

Gerecke R (2002). The water mites (Acari, Hydrachnidia) of a little disturb forest stream in southwest Germany – a study on seasonality and habitat preference, with remarks on diversity

patterns in different geographical areas. In: Bernini F, Nannelli R, Nuzzaci G & de Lillo E (eds), Acarid Phylogeny and Evolution. Adaptations in mites and ticks. Kluwer Academic Publishers: 60-89.

Gerecke R & Schwoerbel J (1991). Water quality and water mites (Acari, Actinedida) in the Upper Danube region, 1959-1984. In: Dusbàbek F & Bukva V (eds), Modern Acarology. Akademia, Prague and SPB Academic Publishing, The Hague, Vol. 1: 483-491.

Keszka S & Raczyński M (2004). Morphological characteristic of dace *Leuciscus leuciscus* (L.) from Krąpiel river (N-W Poland). In: Spurny P (ed.), VIII Czech Ichtiological Conference. Procedings of the International Conference. Brno: 48-52.

Kłosowska M, Bańkowska A & Zawal A (2011). Składanie jaj przez niektóre gatunki wodopójek (Hydrachnidia) z rzeki Krąpieli i jej zbiorników dolinnych. Ogólnopolska Konferencja „Zwierzęta w życiu człowieka" oraz XX Jubileuszowy Zjazd Polskiego Towarzystwa Zoologicznego: 60-65.

Kowalik W (1973). Wodopójki (Hydracarina) Jezior Sosnowickich na Pojezierzu Łęczyńsko-Włodawskim. Ann. UMCS, C, 28(27): 331-351.

Kowalik W (1977). Występowanie i rozmieszczenie wodopójek (Hydracarina) w strefie przydennej jeziora Piaseczno. Ann. UMCS, C, 32(25): 323-344.

Kowalik W (1978). Występowanie wodopójek (Hydracarina) w jeziorach o różnej trofii na Pojezierzu Łęczyńsko-Włodawskim. Ann. UMCS, C, 33(32): 443-468.

Kowalik W (1984). Studia faunistyczno-ekologiczne nad wodopójkami (Hydracarina) południowo-wschodniej Polski. Wydawnictwo AR w Lublinie, Rozprawy Naukowe, Lublin.

Kurzątkowska A & Zawal A (2011). *Sigara dorsalis* (Leach, 1817) (Heteroptera: Corixidae). A new species in Poland and changes in its eastern range extension. Zoologica Poloniae 56: 5-10.

Martin P (1997). Zur Faunistik und Substratpräferenz der Wassermilben (Hydrachnidia, Acari) zweier durch feine mineralische Substrate geprägten Bäche in Schleswig-Holstein. Faun. –Ökol. Mitt., 7: 221-237.

Martin P (2008). Wassermilben (Hydrachnidia, Acari) und Insekten: Ein Überblick über eine selten betrachtete Beziehung. Entomologie heute, 20: 45-75.

Martin P, Dabert M & Dabert J (2010). Molecular evidence for species separation in the water mite *Hygrobates nigromaculatus* Lebert, 1879 (Acari, Hydrachnidia): evolutionary consequences of the loss of larval parasitism. Aquatic Sciences 72 (3): 347-360. http://dx.doi.org/10.1007/s00027-010-0135-x

Meyer E & Schwoerbel J (1981). Untersuchungen zur Phänologie der Wassermilben (Hydracarina) des Mindelsees. Arch. Hydrobiol. (Suppl.) 59(2/3): 192-251.

Pieczyński E (1959). Wodopójki (Hydracarina) niektórych środowisk litoralowych jeziora Tajty oraz innych jezior mazurskich. Ekol. Pol. A, 7(5): 169-198.

Raczyńska M & Machula S (2006). Oddziaływanie stawów karpiowych na jakość wód rzeki Krąpiel (Pomorze Zachodnie). Infrastruktura i Ekologia Terenów Wiejskich, 4: 141-149.

Stępień E, Zawal A, Buczyńska E & Buczyński P (2015). Changes in the vegetation of a small lowland river valley (Krąpiel, NW Poland) after dredging. Acta Biologica, 22: 128-153.

Stoch F, Gerecke R, Pieri V, Rossetti G & Sambugar B (2011). Exploring species distribution of spring meiofauna (Annelida, Acari, Crustacea) in the south-eastern Alps. In: Cantonati M, Gerecke R, Jüttner I & Cox EJ (eds), Springs: neglected key habitats for biodiversity conservation: 65-76. J. Limnol., 70(Suppl. 1), http://dx.doi.org/10.3274/JL11-70-S1-05

Stryjecki R (2002). The impact of human activity on the water mite fauna (Acari, Hydrachnidia) of the „Lasy Janowskie" Landscape Park (South-Eastern Poland). In: Bernini F, Nannelli R, Nuzzaci G & de Lillo E (eds), Acarid Phylogeny and Evolution. Adaptations in mites and ticks. Kluwer Academic Publishers: 113-119.

Stur E, Martin P & Ekrem T (2005). Non-biting midges as hosts for water mite larvae in spring habitats in Luxembourg. Ann. Limnol. - Int. J. Lim., 41(4): 225-236. http://dx.doi.org/10.1051/limn/2005015

Szlauer-Łukaszewska A & Zawal A (2014). The impact of river dredging on ostracod assemblages in the Krąpiel River (NW Poland), 185: 295-305. http://dx.doi.org/10.1127/fal/2014/0620

Van Haaren T & Tempelman D (2009). The Dutch species of *Limnesia*, with ecological and biological notes (Acari: Hydrachnidia: Limnesiidae).

Nederlanse Faunistische Mededelingen, 30: 53-74.

Viets K (1930). Quantitative Untersuchungen über die Hydracarinen der noeddeutschen Seen. Arch. Hydrobiol., 12: 1-71.

Viets K (1931). Tiefenverteilung eniger Hydracarinen in norddeutschen Seen. Zool. Anz., 96: 276-282.

Viets K (1936). Spinnentiere oder Arachnoidea. VII: Wassermilben oder Hydracarina. Tierwelt Dtschl. 31: 1-288, 32: 289-374.

Zawal A (1992). Water mites (*Hydracarina*) of three small lakes in the neighbourhood of Poznań. Acta Hydrobiol., 34: 157-174.

Zawal A (2006). Materiały do znajomości wodopójek (Acari: Hydrachnidia) okolic Złocieńca (północno-zachodnia Polska). Acta Biologica, 13: 163-169.

Zawal A (2007). Wodopójki (Hydrachnidia) rezerwatu „Jezioro Szare" i jego otuliny. Par. Nar. Rez. Przyr., 26(4): 57-78.

Zawal A (2009). Nowe stanowisko *Crocothemis erythraea* (Brullé, 1832) w Polsce zachodniej. Odonatrix 6: 6-8.

Zawal A & Stępień E (2007). Charakterystyka fizyko-chemiczna wód rezerwatu „Jezioro Szare'' oraz ocena jego podatności na degradację. Par. Nar. Rez. Przyr. 26(4): 3-19.

Zawal A, Stępień E, Szlauer-Łukaszewska A, Michoński G, Kłosowska M, Bańkowska A, Myśliwy M, Stryjecki R, Buczyńska E & Buczyński P (2015). The influence of dredging of a lowland river (the Krąpiel in NW Poland) on water mite fauna (Acari: Hydrachnidia), 186: 217-232. http://dx.doi.org/10.1127/fal/2015/0735

Received: March 20th, 2014

Accepted: June 1st, 2015

Branch editor: Stuart Halse

Belg. J. Zool., 145(2) : 103-114 July 2015

Long-term changes of breeding success in Montagu's Harrier *Circus pygargus*

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ABSTRACT. Over a period of almost twenty years, clutch size and breeding success in the Montagu's Harrier were investigated in the context of changing environmental conditions in the species' natural breeding habitats in eastern Poland. During the study periods (1990-95 and 2003-12) a decline was noted in the number of breeding pairs in the population nesting on the calcareous peat bogs near Chełm, not far from the Polish-Ukrainian border. Statistically significant differences in breeding parameters between the two periods were also observed. In the first period clutch volumes were greater, as the dimensions of the individual eggs were larger; additionally, more eggs hatched and the hatchling survival rate was higher. Some habitat conditions were different in the two periods, with the water level and height of vegetation near the nests being lower in the second period. The harriers' food in the two study periods fluctuated strongly with regard to the content of small mammals and compensatory items. In the second period a distinct increase in predator pressure was noted. Pressure from terrestrial predators diminished whereas that from aerial predators increased. Broods in semi-colonies, where birds actively defended their nests, enjoyed a higher rate of survival, as did nests situated far in from the edge of peat bogs. The results suggest that the decline in breeding numbers was driven by increased predation, which was in turn a consequence of habitat changes in the natural environment of eastern Poland.

KEY WORDS: *Circus pygargus*, breeding success, predation, habitat changes

INTRODUCTION

The Montagu's Harrier *Circus pygargus* is a medium-sized raptor nesting in farmland (Cramp & Simmons, 1980, 2000; Clarke, 1996; Arroyo et al., 2004) and in natural wetlands and peat bogs, the latter particularly in eastern Europe (Krogulec & Leroux, 1994; Wiącek, 2006, 2009). In eastern Poland, declines in populations and breeding success of Montagu's Harrier have been observed in recent decades (Wiącek, 2007). The species shows a tendency to nest semi-colonially throughout its range (Arroyo et al., 2004; Wiącek, 2006b, 2008; Kitowski, 2008; Krupiński et al., 2010). The reproductive success of females in many bird species is partly determined by clutch size and egg size (Blackburn, 1991), and is influenced by food supply (Stearns, 1992; Arroyo, 1998; Arroyo & Garcia 2006; Koks et al., 2007). The availability of food and its fluctuations even during a single season can seriously affect breeding success (Tremblay et al., 2003). Studies on the effect of food on clutch size in Montagu's Harriers confirm this dependence (Salamolard et al., 2000; Millon et al., 2008): for example, young or poorly fed females lay fewer and smaller eggs (Salamolard,1998, Arroyo et al., 2004; Arroyo et al., 2007; Millon et al., 2008). The dependence between food abundance and breeding success is particularly conspicuous in vole-eating predators (Korpimaki, 1990; Brommer et al., 2002).

Being a ground nesting raptor, the Montagu's Harrier is itself vulnerable to predation (Clarke, 1996; Simmons, 2000). Harrier nests – usually situated on the edges of marshes in the natural environment of eastern Poland – are easily detected by terrestrial or aerial predators

(Wiącek, 2007, 2009). Another form of predation pressure is intraguild predation (Sergio & Hiraldo, 2008; Quinn et al., 2008). One way of avoiding or decreasing the predation risk is to breed in semi-colonies (Arroyo et al., 2001, 2004; Wiącek, 2008). Many studies confirm that nesting aggregation is advantageous to breeding success in many avian species (Bertram, 1978; Quinn & Ueta, 2008). Mobbing behaviour is another means of enhancing brood safety in a semi-colony (Arroyo et al.,2001; Kitowski, 2004; Wiącek, 2008). The benefits of this behaviour are evident, because they decrease the predation risk and increase breeding success (Birkhead & Moller, 1992; Brown & Brown, 1996).

A further reason for the decline of harriers may be changes to the wintering habitats in the Sahel and mortality during migration and overwintering. The large-scale conversion of floodplain habitat into desiccated grasslands may lead to decreasing food resources and to sub-optimal environmental conditions for wintering harriers (Liminana et al., 2007; Buij et al., 2012). Changes in climate or land use in wintering areas are important for the survival of harrier species (Liminana et al., 2012). All these limiting factors may have contributed to a distinct decline in the numbers of this raptor nesting on peat bogs in eastern Poland.

The main objective of this paper was to analyse the changes in the breeding parameters of Montagu's Harriers during the last two decades on the calcareous peat bogs near Chełm in eastern Poland, in the context of environmental changes, fluctuating food resources and predator pressure.

STUDY AREA AND METHODS

Montagu's Harriers were monitored on the calcareous peat bogs (4309 ha) near Chełm in eastern Poland (51°10' N, 23°37' E). The study area is part of a Special Protection Area for birds within the NATURA 2000 network, located near the Polish-Ukrainian border (Wilk et al., 2010). The dominant vegetation type is the sedge association based on *Cladietum marisci*. There the Harriers build their nests in clumps of sedges surrounded by water, or in partly paludine areas (Wiącek, 2009). The study area was surrounded by farmland, which constituted the foraging habitat of the harriers (Wiącek, 2006a).

The fieldwork was conducted during two periods, i.e. 1990-95 and 2003-12. Montagu's Harrier nests were mapped and monitored frequently (two or three times a week) from egg laying to fledging (from late April to the end of July). Observations started in the pre-laying period in mid-April. In total, 106 nests with complete clutches were found. Replacement clutches were excluded from the study. All nests were observed before being inspected. The laying date was estimated according to the method described by Arroyo (2002). If a few eggs were found in the nest, it was assumed that an interval of 2 days had elapsed between the laying of consecutive eggs (Arroyo et al., 2004). Eggs from 94 nests were individually marked in laying sequence and their lengths and widths measured (n=405, with callipers to the nearest 0.1 mm). 161 eggs were measured in the first period and 244 in the second. Egg volume was calculated with Hoyt's formula (1979): 0.51 x length x width^2.

Weather data for May were analysed, when harriers started incubating, and all nests were found. The mean temperature during egg laying, maximum and minimum temperature, number of days with rainfall, and wind speed were obtained from www.TuTiempo.net, based on the nearest weather station at Lublin-Radawiec airport. The data given here on the composition of food are derived from several other studies conducted in the same study area: Tabor & Tabor (2005, 262 prey items collected during the incubation and nestling periods), Wiącek & Niedźwiedź (2005, 210 prey items collected during the pre-laying period), Wiącek & Niedźwiedź (2009, 618 prey items) and Zietek (2009, 967 prey items collected during the incubation and nestling periods). In the papers cited above, the

Montagu's Harrier diet was determined on the basis of prey remains in the nests and pellet analysis (TABOR & TABOR, 2005; WIĄCEK & NIEDŹWIEDŹ, 2009; ZIĘTEK, 2009) or from the pellets and observation of birds carrying prey during food transfer WIĄCEK & NIEDŹWIEDŹ (2005). Pellets were collected during nest or perch inspections (two or three times a week).

To assess the effect of predation on Montagu's Harrier clutches, 78 nests of the 106 found were closely monitored: 30 in the first study period and 48 in the second. In the first period 15 adult Montagu's Harriers were caught and individually marked with coloured wing tags (KOCHERT et al., 1983; WIĄCEK, 2008). The presence of young females in the study area was determined from feathers remaining in the nests or in flight by direct observation (MILLON et al., 2008).

Clutch survival was defined as the number of days between the laying of the first egg and the last inspection, when at least one hatchling was alive in the nest. Most of the fieldwork carried out near the Harrier nests was based on the methods described by TYLER et al. (1998) with modifications described by WIĄCEK (2009). Vegetation height, depth of water, internal and external nest diameter were measured accurate to 1 cm in mid-May. Vegetation density was measured near the nest at a distance of 0.5 m in plots of 0.1 m^2. In the second study period additional measurements of vegetation density were made at a distance of 2 m from the nests. The numbers of plants were counted along a 1 m section, 0.5 m above the ground in a few randomly chosen spots in the vicinity of the nest. The measurements were averaged for each nest.

Categorisation of Montagu's Harrier nests as clumped or solitary was based on a behavioural criterion described by WIĄCEK (2008). The distance between nests and the nearby meadows was measured with a tape or GPS receiver. The harriers' brood predators were determined from observations conducted near the nest, tracks (footprints) left near the nest, remains of the victims (bite marks on the feathers or eggs in the nest) or by using digital trail cameras (www.ecotone.pl). Four cameras were used in the last two study seasons.

Data analyses were done using logistic regression and nonparametric statistics (Mann-Whitney test, Kruskal-Wallis test and Spearman correlation). All analyses were carried out using Statistica 8.1. The study was conducted with the permission of the Local Ethics Committee for Animal Experimentation and the Regional Directorate for Environmental Conservation in Lublin.

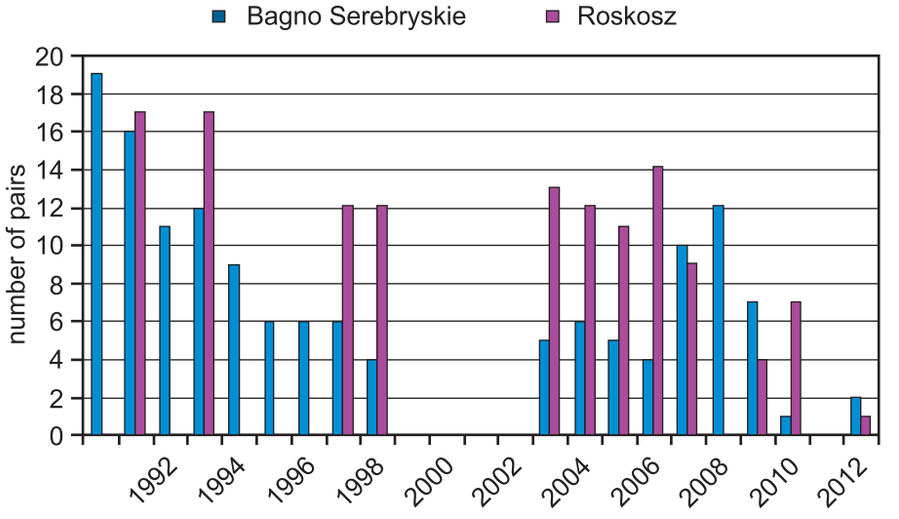

Fig. 1. – Numbers of breeding pairs in two nature reserves in the study area.

RESULTS

Number of breeding pairs

The observations conducted from 1990 to 1998 and from 2003 to 2012 show a significant decrease in the number of pairs nesting in the two peat bog reserves that were studied (Fig. 1): from 17 to 1 pair in the "Roskosz" reserve and from 19 to 2 pairs in the "Bagno Serebryskie" reserve (Table 2).

Eggs, chicks and fledglings

The mean egg volume and the mean clutch volume per female were both larger in the first period than in the second one. There were significant differences between egg volumes in the two study periods (Table 2). The mean dimensions of eggs laid in the 1990s were greater (4.25 x 3.37 cm) than in the second period (4.17 x 3.35 cm). The differences in egg length and width in the two study periods were statistically significant (Table 2). The first and last eggs in a clutch were smaller than those laid in the middle of the sequence (Fig. 2). In the 1990s, 3-, 4- and 5-egg clutches were reported (n=37), whereas after 2003 (n=66), one small clutch of 2 eggs and 2 clutches of 6 eggs were also recorded. The mean clutch size was similar in both periods (Table 2). In the first study period females started laying eggs two days earlier than during the second one. Nonetheless, the overall timing of egg laying did not differ statistically (Table 2). The numbers of chicks hatched were different in the two study periods; in the first, the mean number of nestlings hatched was higher than in the second. The numbers of fledglings in the two study periods were also different: in the first period, the mean number of fledglings in all nests was higher than in the second one (Table 2).

Nest and environmental factors

The nests built by the harriers in the two study periods differed in size. The mean diameter of nests built in the 1990s was smaller than that of the nests in the second period (Table 2). The diameter of the nest was not related to brood size (Mann-Whitney test z=1.603, n=78, p=0.87) or clutch size, but the relationship between diameter and clutch size was near-significant (Mann-Whitney test z=1.94, n=78, p=0.052). Weather conditions in May, when most harriers started incubating were similar in both study periods. There were no differences between them with respect to the following weather parameters: maximum temperature (Mann-Whitney test z=-0.96, n=14,

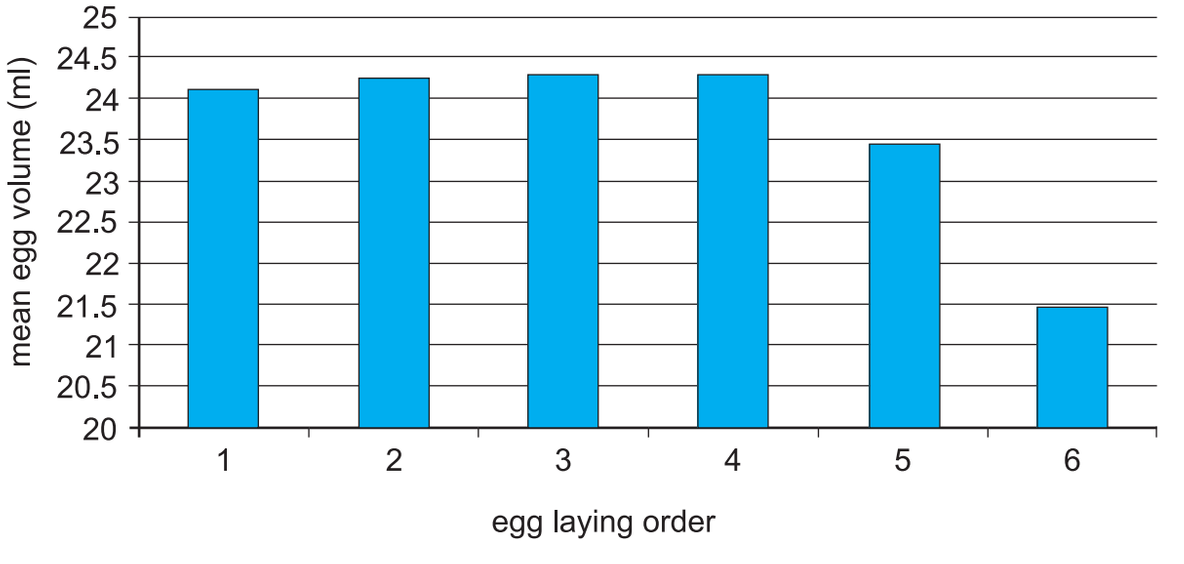

Fig. 2. – Mean egg volume vs. egg laying order.

Table 1

Changes in food resources of the Montagu's Harrier in the study area (a,d - Wiącek & Niedźwiedź 2009; b-Tabor & Tabor 2005; c-Wiącek & Niedźwiedź 2005, e-Ziętek 2009).

Food categories (%)	Period				
	1985-89 (a)	1988-89 (b)	1992-95 (c)	2004-08 (d)	2007 (e)
mammals	64.2	36.3	56.2	30.3	18.2
birds	27.2	22.5	24.3	33.3	4
birds' eggs	1.1	0	9.5	3	0
amphibians	0.2	0	1.4	0	0
reptiles	1.9	0	8.1	16.2	3.7
invertebrates	5.4	41.2	0	17.2	74
% of Common Vole in mammalian prey	55	29.5	50	23	69

P=0.33), minimum temperature (z=-1.67, n=14, P=0.09), number of days with rainfall (z=-1.69, n=14, P=0.09) and wind speed (z=0.77, n=14, P=0.43). The mean temperature in both periods was also similar (Mann-Whitney test z=-1.42, n=14, P=0.15). The water level on the peat bog where the Montagu's Harriers built their nests was different in the two study periods. During the first period (1992-95) the mean water level was lower than in the second one (Table 2). The mean height of the vegetation near the nests as measured from the nest base were different in the two study periods, being higher in the first period than in the second one. The differences between the two periods were near-significant (Table 2). In 2008-09, the density of the vegetation directly adjoining the nests was measured. Both the nests in semi-colonies (mean density 98.2/0.1m^2, SD=14.95, n=10) and the isolated ones (mean density 97.7/0.1m^2, SD=12.78, n=10) had been built in vegetation patches of similar density (Mann-Whitney test z=0.468, P=0.63, n=20). Investigations of vegetation density at a distance of 2 m from the nest did reveal differences, however (Mann-Whitney test z=2.114, P=0.033, n=20). Measurements in semi-colonies indicated that these nests had been built in larger patches of dense vegetation (on average 24 plants in a 1 m^2 section, SD=4.32, n=10) than were the isolated nests (on average 19.3, SD=5.33, n=10).

Analysis of the food composition in the two study periods (Table 1) reveals strong fluctuations in the numbers of small mammals, birds, reptiles and invertebrates in the harriers' diet. The percentage of common vole (*Microtus arvalis*) in the total mammalian prey also fluctuated strongly.

Brood losses

During the two study periods, 40 of the 78 Montagu's Harrier broods monitored were destroyed by predators and three others were lost for different reasons – in one case the nest was flooded and in the other two the eggs were addled. In the first study period in the 1990s, 20% of broods were destroyed by predators. The perpetrator in five cases was a predatory mammal, probably a fox, and in the sixth case it was a Marsh Harrier. In the second study period, 75% of broods were destroyed (Table 2). The predators in these cases were corvids, which destroyed 19 (52%) broods, Marsh Harriers – 9 (25%) and foxes or other mammals – 6 (16%). All the eggs in two clutches turned out to be addled (5%) and one nest with eggs was flooded following very heavy rainfall (2%). During the first study period, there were also partial losses in 21 successful broods, from which at least one

Table 2

The main results.

	First period	Second period	Differences
Number of breeding pairs	R=0.67; Beta= -0.67; n=19; p=0.001	R=0.84; Beta= -0.84; n=12; p=0.006	Significant decrease in both study periods
Nest diameter	Φ=31.46; SD=53.37 n=30	Φ=34.78; SD=37.32; n=48	Mann-Whitney test z=2.59; n=78; p=0.009
Mean egg volume	24.81; SD=1.85;; n=161	23.74; SD=2.16; n=244	Mann-Whitney test z=5.71; n=161+244; p<0.0001
Mean clutch volume per female	109.27; SD=13.53, n=37	93.53; SD=24.79; n=55	Kruskal-Wallis test H=8.08; n=92; p=0.004
Mean dimension of eggs	4.25 x 3.37 cm;n=161, SD_{length}=0.16, SD_{width}=0.09	4.17 x 3.35 cm; n=244, SD_{length}=0.19, SD_{width}=0.12	Mann-Whitney test (length) z=-2.86, n=92, P=0.004 Mann-Whitney test (width) z=-2.90, n=92, P=0.004
Mean clutch size	4.36; SD=0.54; n=37	4.22; SD=0.79; n=66	Mann-Whitney test z=0.72; n=103; p=0.42
First egg (laying date)	16th of May, 15.38, SD 3.39; n=37	18th of May, 17.36; SD=7.76; n=55	Kruskal-Wallis test H=19.37; n=92; p=0.08
Chicks hatched	3.39; SD=1.33, n=33	2.01; SD=1.88; n=73	Mann-Whitney test z=3.104; n=106; p=0.001
Number of fledglings	2.23; SD=1.1; n=30	0.45; SD=0.95; n=73	Mann-Whitney test z=5.61; n=103; p<0.0005
Water level	3.16cm; SD=2.8; n=30	15.01cm; SD=1.89; n=48	Mann-Whitney test z=5.09; n=78; p=0.0001
Vegetation height	85.16 cm; SD=10.88; n=30	72.4 cm; SD=14.29; n=48	Mann-Whitney test z=1.92; n=78; p=-0.052
Brood losses	20% (6 from 30)	75% (37 out of 48)	
Brood survival	53.3 days SD=42.68; n=30	40.73 days SD=3.51; n=41	Mann-Whitney test z=-3.69; n=71; p=0.0002
Brood survival in a semi-colony or in solitary nests	In a semi-colony 59.36 days SD=2.67; n=19. Solitary nests: 42.9; SD=23; n=11	In a semi-colony: 45.86; SD=16.7; n=30. Solitary nests 26.72 SD=9.85; n=11	First: Mann-Whitney z=1.84; n=30; p=0.06 Second: Mann-Whitney z=3.29; n=41; p=0.0009

nestling fledged. The causes of mortality in the 32 chicks that died were starvation (30 chicks – 94%) and sibling cannibalism (2 chicks – 6%). In addition, six eggs were addled. In the second period, partial losses were recorded in 11 successful broods. Then, the causes of mortality in 20 chicks were starvation (11 chicks – 55%), predation by Marsh Harriers (4 chicks – 20%), sibling cannibalism (3 chicks – 15%), drowning (1 chick- 5%), trampling by wild boar (1 chick – 5%); three eggs were addled.

Brood survival was higher in the 1990s than after the year 2000 (Table 2). In the first period most

of the nests (5 out of 6) destroyed by predators were situated outside the semi-colonies. The time elapsing between the construction of a nest in a semi-colony to its destruction by a predator was longer than if it was isolated, but the differences were not statistically significant (Table 2). In the second period (after 2000), when predator pressure was greater, nests in semi-colonies had a far greater chance of survival than nests built in isolation (Table 2).

In the first period, when water levels near the nests were low, predators destroyed nests situated closed to the edge of the peat bog. This relationship was statistically significant (Mann-Whitney test: z=2.48, P=0.012, n=30). In most cases (5) the predator was a fox or other mammal; only once was a Marsh Harrier the culprit. In the second period, when water levels were higher and it was generally harder for terrestrial predators to gain access to the nests than in the first period, no such relationship could be discerned (Mann-Whitney test: z=1.004, P=0.3, n=48). Interestingly, the water levels in the first period were similar around nests that were successful and those that failed (Mann-Whitney test, z=-0.72, P=0.45, n=30). The critical factors determining the success of a nest and enabling attacks by predators to be foiled was the distance from meadows and nesting in a semi-colony (Table 3). Other parameters measured in the study area did not have any serious effect on brood survival in either of the study periods.

DISCUSSION

Breeding success

Egg sizes in the two study periods were significantly different. In the first one, the size of eggs (42.5 x 33.7 mm) was to my knowledge the largest described in the literature. In the second period, egg sizes (41.7 x 33.5 mm) were similar to those given by other authors monitoring harrier nests (Arroyo et al., 1998; Corbacho & Sanchez, 2000; Arroyo et al., 2004). The differences in egg size between the study periods were probably the result of better food conditions during the first one; associations between the availability of food and breeding parameters of Montagu's Harriers have been found before (Salamolard et al., 2000; Millon et al., 2008). Alternatively, the smaller egg size in the second study period could have been due to a larger number of young females starting to breed in the second period, because young females produce significantly smaller eggs in comparison with older females (Arroyo & Garcia, 2006; Arroyo et al., 2007).

Clutch sizes in the Montagu's Harrier populations studied here were similar to the mean results from Europe cited by Cramp & Simmons (1980). They were distinctly larger than in the majority of studies reported from Italy and France. At some sites, clutch sizes were similar to our study, for example, in Charente-Maritime and Deux Sèvres in France or in England. However, in some places in Spain and Portugal these values were lower (Arroyo et al., 2004). The numbers of birds hatching in the two periods we studied were comparatively high, but the number of hatchlings was significantly lower in the second period than in the first one. These differences were due to greater predation pressure in the second study period. Nevertheless, both values were similar to the data obtained in France (Arroyo et al. 2004), the Netherlands (Schipper, 1979), Spain (Arroyo et al., 2004) and England (Underhill-Day, 1990).

Food

During the two study periods, there were strong fluctuations in the availability of harrier food. The changes in the proportions of small mammals reflected the typical periodic fluctuations in the numbers of these animals (Lambin et al., 2006; Liminana et al., 2012a). In the periods when small mammals predominated in the harriers' diet (Table 1) the proportion of other prey items decreased. In other periods, when mammalian food was not readily available, the proportion of other prey items compensating for the lack

Table 3

Probability of breeding success in harriers explained by different variables (forward stepwise logistic regression). Only significant variables are shown.

Variable	Estimate	SE	T	P
Constant	4.672	-12.419	-2.658	0.009
Distance to the meadows	1.485	0.015	2.021	0.046
Nesting in semi-colony	1.485	3.935	2.649	0.009

of small mammals, such as reptiles, birds or invertebrates, increased. According to the ornithological literature, mammals and birds are the most important components of the Montagu's Harrier diet (Arroyo et al., 2004; Terraube & Arroyo 2011; Liminana et al. 2012a). This high-energy food is easier to assimilate than other items (Tollan, 1988). A link between the availability of common voles and the breeding parameters of Montagu's Harriers was demonstrated by research done in France (Salamolard et al., 2000; Millon et al., 2008). Analogous relationships were also found for owls (Korpimaki, 1990) and kestrels (Wiebe & Bortolotti 1994; Wiehn & Korpimaki 1997). The proportion of common voles in the harrier diet, recorded in France, fluctuated between 33 and 86% (Millon et al., 2008), values that are similar to those we obtained in our study area (Table 1). However, in comparison with other factors such as predation or age of the females, the food fluctuations observed in the study area in both study periods may have been less important for breeding success.

Predator pressure

The number of fledglings was different in the two study periods, decreasing significantly from 2.5 per successful nest in the first to 1.9 in the second period (from 2.3 to 0.45 in all monitored nests). The main factor limiting the number of fledglings was predator pressure. In the second study period, the mean length of time during which a nest was active was almost two weeks shorter than in the first period. Changes in predator pressure were due to mammalian predators (probably red foxes) and corvids during the incubation stage, and increasing predator pressure from Marsh Harriers in the nestling period (Wiącek, 2007). The main reason for the increase in the red fox population has been the nationwide anti-rabies vaccination programme in Poland, which started just before 2000. The increasing pressure from corvids on Montagu's Harrier broods is due to the high density of Magpies and Ravens breeding in Poland in both study periods (Jerzak, 2005, Bednorz, 2005, Panek, 2005, www.monitoringptakow.gios.gov.pl). An important factor facilitating access to harrier nests in the first study period was the low water level. In such conditions it was quite easy for terrestrial mammalian predators, mostly foxes, but also cats and feral dogs, to gain access to the nests (Tryjanowski et al., 2002, 2009). The first nests to be destroyed were those situated at the edge of the peat bog. Therefore, the distance between the meadows around the marshes and the harrier nests was important for breeding success in both periods. In the second period, the water level was far higher, so access to the nests was much more difficult. As a consequence, nest losses due to terrestrial predators were fewer, but against that there was much greater pressure on the nests from aerial predators like Marsh Harriers and corvids (Wiącek, 2007). The losses caused by predators also depended on other habitat factors. One of these, enabling predators to discover nests in the second period, was the shorter vegetation close to the harriers' nests: this did not provide sufficient cover for incubating

females (Arroyo et al., 2004, Liminiana et al., 2006; Wiącek, 2009). However, the most important factor in the destruction of Montagu's Harrier broods was intraguild predation and the presence of breeding Marsh Harriers in the same area (Buczek & Keller 1994; Wiącek, 2005; Sergio & Hiraldo 2008).

One factor significantly modifying breeding success in the Montagu's Harriers was their nesting in semi-colonies or in isolation. Brood losses from colonial and isolated nests differed significantly: colonial breeding was far safer (Arroyo et al., 2001; Wiącek, 2008). All the nests, whether in semi-colonies or in isolation, were built in patches of denser vegetation (Wiącek, 2009), but the semi-colonial nests were situated in larger patches of optimal habitat. In heterogeneous natural habitats such as bogs or marshland, the size of available patches is more important for semi-colony formation than in fields, which are large, homogeneous habitats. The formation of a semi-colony in a field is probably behaviour-based, since the availability of optimal habitat offering a secure nest site is greater than in a structurally heterogeneous, natural peat bog. Evidence for this is provided by the greater distances between semi-colonial nests in fields in Spain and France than on peat bogs in eastern Poland (Arroyo et al., 2001; Wiącek, 2008). On a peat bog, the "capacity" of the optimal nesting habitat is limited, hence the greater density of nests in semi-colonies. In both variants, the basic factors as regards nesting are the availability of food in the vicinity of the semi-colony and nest security (Arroyo et al., 2001; Wiącek, 2008, 2009). Additionally, the active conservation of some rare bird species, such as Aquatic Warbler *Acrocephalus paludicola* living in the same habitats as the Montagu's Harrier, has contributed to the destruction of the optimum structure of the nesting habitat utilised by this raptor. While mowing the tall vegetation growing on the peat bog optimises the habitat for some species, it destroys the habitat for other species with diametrically opposed habitat requirements (cranes, harriers or bitterns).

CONCLUSIONS

In the 1980s the study area boasted the greatest density of breeding Montagu's Harriers in Europe (Krogulec & Leroux, 1994). Observations conducted in this area over two study periods showed a decrease in the number of pairs nesting there (Fig. 1). This was driven by changes to their traditional breeding habitat (higher water level, shorter vegetation, mowing or burning of sedge beds). This led to a deterioration in a whole range of breeding parameters, not to mention a rapid increase in predation pressure and strong fluctuations in food availability in the study area.

ACKNOWLEDGEMENTS

I am grateful to Beatriz Arroyo and the anonymous referees and editors for their useful suggestions, advice and comments. Peter Senn reviewed the English version of this paper.

REFERENCES

Arroyo B (1998). Effect of diet on the reproductive success of Montagu's Harrier *Circus pygargus*. Ibis 140: 690-693.

Arroyo B (2002). Sex-biased nestling mortality in the Montagu's Harrier *Circus pygargus*. J. Avian Biol. 33: 455-460.

Arroyo B & Garcia J (2006). Diet composition influences annual breeding success of Montagu's harrier *Circus pygargus* feeding on diverse prey. Bird Study 53: 73-78.

Arroyo B, Leroux A & Bretagnolle V (1998). Patterns of egg and clutch size variation in Montagu's Harrier. Journal of Raptor Research 32: 136-42.

Arroyo B, Mougeot F & Bretagnolle V (2001). Colonial breeding and nest defence in Montagu's Harrier. Behav. Ecol. Sociobiol. 50: 109-115.

Arroyo B, Garcia JT & Bretagnolle V (2004). Montagu's Harrier. BWP Update 6: 39-53.

Arroyo B, Bretagnolle V & Leroux A (2007). Interactive effects of food and age on breeding in the Montagu's Harrier *Circus pygargus*. Ibis 149: 806-813.

Bednorz J (2005). Current knowledge about the Raven *Corvus corax* in Poland. In: Jerzak L, Kavanagh B & Tryjanowski P (eds), Corvids of Poland, pp.127-135, Bogucki Wydawnictwo Naukowe, Poznań.

Bertram B (1978). Living in groups: predators and prey. In: Krebs J & Davies N (eds), Behavioural ecology: an evolutionary approach, pp. 64-97, Oxford.

Birkhead T & Moller A (1992). Sperm competition in birds. Academic Press, London.

Blackburn T (1991). An interspecific relationship between egg size and clutch size in birds. Auk 108: 209-211.

Brommer JE, Pietianen H & Kolunen H (2002). Reproduction and survival in a variable environment: Ural owls (*Strix uralensis*) and the three-year vole cycle. Auk 119: 544-550.

Brown C & Brown M (1996). Coloniality in the cliff swallows. University of Chicago Press, Chicago.

Buczek T & Keller M (1994). Breeding ecology of the Marsh harrier *Circus aeruginosus* in eastern Poland. Population numbers and phenology of the onset and laying. Acta Ornithologica 29: 67-80.

Buij R, Van der Goes D, De Jongh H, Gagare S, Haccou P, Komdeur J & De Snoo G (2012). Interspecific and intraspecific differences in habitat use and their conservation implications for Palearctic harriers on Sahelian wintering grounds. Ibis 154: 96-110.

Clarke R (1996). Montagu's Harrier. Arlequin Press, Chelmsford.

Corbacho C, Sanchez J & Sanchez A (1997). Breeding biology of Montagu's harrier *Circus pygargus* in agricultural environments of southwest Spain; comparison with other populations in the western Palearctic. Bird Study 44: 166-175.

Cramp S & Simmons KEL (1980). The birds of the western Palearctic, vol. 2. Oxford University Press, Oxford.

Hoyt D (1979). Practical methods of estimating volume and fresh weight of birds eggs. Auk 96: 73-77.

Jerzak L (2005). Magpie *Pica pica* in Poland – current state of knowledge. In: Jerzak L, Kavanagh B & Tryjanowski P (eds), Corvids of Poland, pp. 35-51, Bogucki Wydawnictwo Naukowe, Poznań.

Kitowski I (2004). Behaviour of Red Foxes *Vulpes vulpes* during of Montagu's harrier *Circus pygargus* social defences – case study from southeast Poland. Acta Biol. Univ. Daugavp. 4(2): 71-76.

Kitowski I (2008). Breeding ecology of Montagu's Harrier *Circus pygargus* in marshes of eastern Poland: importance of aggregated nesting. Acta Zool. Lituanica 18(2): 83-89.

Kochert M, Stenhoof K & Mortish K (1983). Evaluation of patagial markers for raptors and ravens. Wildlife Society Bulletin 11: 271-281.

Koks B, Trierweiler K, Visser E, Dijkstra C &. Komdeur J (2007). Do voles make agricultural habitat attractive to Montagu's Harrier *Circus pygargus*? Ibis149: 575-586.

Korpimaki E (1990). Low repeatability of laying date and clutch size in Tengmalm's owl: an adaptations to fluctuating food conditions. Ornis Scand. 21: 282-286.

Krogulec J & Leroux A(1994). Breeding ecology of Montagu's Harrier *Circus pygargus* on natural and reclaimed marshes in Poland and France. In: Meyburg B & Chancellor R (eds), Raptor Conservation Today, pp. 151-152, Proceedings of the IV World Conference on Birds of Prey and Owls. Berlin, Germany, 10-17 May 1992, Pica Press, London.

Krupiński D, Lewtak J & Szulak K (2010). Semicolonial nesting and conservation of the Montagu's harrier *Circus pygargus* in rapeseed fields in Southern Podlasie (eastern Poland). Slovak Raptor Journal 4: 37-40.

Krupiński D, Lewtak J, Rzępała M & Szulak K (2012). Breeding biology of the Montagu's harrier *Circus pygargus* in east-central Poland and implications for its conservation. Zoology and Ecology, 22(2): 86-92.

Lambin X, Bretagnolle V & Yoccoz G (2006). Vole population cycles in northern and southern Europe: is there a need for different explanations for single pattern. J. Anim. Ecol. 75: 340-349.

Liminana R, Soutullo A, Urios V & Surroca M (2006). Vegetation height selection in Montagu's Harriers *Circus pygargus* breeding in a natural habitat. Ardea 94: 280-284.

Liminana R, Soutullo A & Urios V (2007). Autumn migration of Montagu's harriers *Circus pygargus* tracked by satellite telemetry. J. Ornithol 148: 517-523.

Liminana R, Soutullo A, Urios V & Reig-Ferrer A (2012). Migration and wintering areas of adult Montagu's Harriers *Circus pygargus* breeding in Spain. J Ornithol 153: 85-93.

Liminana R, Javaloyest & Urios V (2012a). Diet of the Montagu's Harrier *Circus pygargus* nesting in natural habitat in Eastern Spain. Ornis Fennica 89: 74-80.

Millon A, Arroyo B & Bretagnolle V (2008). Variable but predictable prey availability affects predator breeding success: natural versus experimental evidence. Journal of Zoology 275: 349-358.

Panek M (2005). Activity of the Raven *Corvus corax* on farmland and an assessment of its predation on Brown Hares *Lepus europaeus* near Czempiń. In: Jerzak L, Kavanagh B & Tryjanowski P (eds), Corvids of Poland, pp. 407-418. Bogucki Wydawnictwo Naukowe, Poznań.

Quinn J& Ueta M (2008). Protective nesting associations in birds. Ibis 150: 146-167.

Quinn J, Renolds J & Bradbury R (2008). Birds as predators and as prey. Ibis 150: 1-8.

Salamolard M (1998). Stratégie d'utilisation des ressources chez une espèce de rapace semi-colonial, le busard cendre (*Circus pygargus*). PhD thesis, Université de Tours.

Salamolard M, Butet A, Leroux A & Bretagnolle V (2000). Responses of an avian predator to variations in prey density at a temperate latitude. Ecology 81: 2428-2441.

Schipper W (1979). A comparison of breeding ecology in three European harriers (*Circus*). Ardea 66: 77-102.

Sergio F & Hiraldo F (2008). Intraguild predation in raptor assemblages: a review. Ibis 150: 132-145.

Simmons R (2000). Harriers of the World. Oxford University Press, New York, United States.

STATSOFT INC(2001). Statistica for Windows (data analysis system).

Stearns S (1992). The evolution of life history. Oxford University Press, Oxford.

Tabor M & Tabor J (2005). Food of the Montagu's harrier *Circus pygargus* in the breeding period on the Calcareous Marshes near Chełm. Kulon 10: 33-42 (in Polish).

Terraube J & Arroyo B (2011). Factors influencing diet variation in a generalist predator across its range distribution. Biodivers. Conserv. 20: 2111-2131.

Tollan A (1988). Maintenance energy requirements and energy assimilation efficiency of the Australasian Harrier. Ardea 76: 181-186.

Tremblay I, Thomas D, Lambrechts M, Blondel J & Perret P (2003). Variations in blue tit breeding performance across gradients in habitat richness. Ecology 84: 3033-3043.

Tryjanowski P, Gołdyn B & Surmacki A (2002). Influence of the red fox *Vulpes vulpes* on the distribution and number of breeding birds in an intensively used farmland. Ecol. Res. 17: 395-399.

Tryjanowski P, Kuźniak S, Kujawa K & Jerzak L (2009). Ekologia ptaków krajobrazu rolniczego. Bogucki Wydawnictwo Naukowe, Poznań (In Polish).

Tyler G, Smith K & Burges D (1998). Reedbed management and breeding Bitterns *Botaurus stellaris* in UK. Biol. Conserv. 86: 257-266.

Underhill-Day J (1990). The status and breeding biology of Marsh Harrier and Montagu's Harrier in Britain since 1900. PhD thesis, CNAA, London, UK.

Wiącek J (2006). The fluctuation in the number of Montagu's Harrier *Circus pygargus* in the context of the change in the protection status and utilization methods on the territory of Chełm's calcareous peat bogs. Polish J. Environ. Stud. 15 (5d): 737-741.

Wiącek J (2006a). Food transfer in Montagu's Harrier *Circus pygargus* during the courtship. Acta Ornithologica 41: 88-91.

Wiącek J (2006b). Behavioural consequences of semi-colonial breeding in Montagu's Harrier in Poland. Journal of Ornithology 5: 271. XXIV International Ornithological Congress, Hamburg.

Wiącek J (2007). Nest predation in the Montagu's Harrier *Circus pygargus* in natural habitats of Eastern Poland. In: Birds as Predators and as Prey. BOU Annual Spring Conference 2007, University of Leicester, England.

Wiącek J (2008). Benefits and costs of semi-colonial breeding in the Montagu's Harrier *Circus pygargus*. Belg. J. Zool. 138(1): 36-40.

Wiącek J (2009). Nest site selection of Montagu's Harrier *Circus pygargus* breeding in natural habitats in eastern Poland. Ardea 97(1):117-119.

Wiącek J & Niedźwiedź M (2005). The food of Montagu's Harrier *Circus pygargus* in the pre-laying period. Berkut, 14/2:189-192.

Wiącek J & Niedźwiedź M (2009). Long term changes in the diet of Montagu's Harrier *Circus pygargus* during the nesting period on the Chełm Calcareous Marshes. In: Wiącek J, Polak M, Kucharczyk M, Grzywaczewski G& Jerzak L (eds), Birds-Environment-Threats-Conservation. Selected issues in avian ecology, pp. 309-318. LTO, Lublin, Poland (In Polish, with English summaries).

Wiebe K & Bortolotti G (1994). Food supply and hatching spans of birds: energy constraints or facultative manipulation? Ecology 75: 813-823.

Wiehn J & Korpimaki E (1997). Food limitation on brood size: experimental evidence in the Eurasian kestrel. Ecology 78: 2043-2050.

Wilk T, Jujka M, Krogulec J & Chylarecki P (2010). Important Bird Areas of international importance in Poland. OTOP, Marki. www.monitoringptakow.gios.gov.pl

Ziętek K (2009). Food composition of Kestrel *Falco tinnunculus* and Montagu's harrier *Circus pygargus* in eastern Poland. MSc manuscript, Department of Nature Conservation, Curie-Skłodowska University, Lublin, Poland (in Polish).

Received: April 11th, 2014

Accepted: March 18th, 2015

Branch editor: Diederik Strubbe

Belg. J. Zool., 145(2) : 115-129 July 2015

Complete mitochondrial genome of *Whitmania laevis* (Annelida, Hirudinea) and comparative analyses within *Whitmania* mitochondrial genomes

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ABSTRACT. The complete mitochondrial genome of *Whitmania laevis* is 14,442 bp in length and contains 37 genes including 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, and two ribosomal RNA (rRNA) genes. The almost-complete mitochondrial genome of *Whitmania acranulata*, consisting of 13,494 bp, contains 35 genes including 13 PCGs, 20 tRNA genes, and two rRNA genes. COI phylogenetic analyses showed that the samples reported in GenBank and analysed as *Hirudo nipponia* KC667144, *Hirudinaria manillensis* KC688268 and *Erpobdella octoculata* KC688270 are not the named species and they should belong to *Whitmania*. We compared and analyzed the characteristics of nucleotide composition, codon usage, and secondary structures of 22 tRNAs and two rRNAs from *Whitmania* taxa. Moreover, we analyzed phylogenetic relationships of Annelida using maximum likelihood (ML) and Bayesian inference (BI) methods, based on 11 mitochondrial genes. Our results reveal that *W. laevis* has a close relationship with *W. pigra*.

KEY WORDS: *Whitmania laevis*, *Whitmania acranulata*, mitochondrial genome, comparative analyses, phylogenetics

INTRODUCTION

The typical metazoan mitochondrial genome is a double-stranded circular DNA molecule, varying in length from 14 to 20 kb, usually composed of 36–37 genes including 12–13 protein-coding genes (PCGs), two ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes (Boore, 1999). The mitochondrial genome is becoming increasingly important for phylogenetic reconstruction, due to its rapid evolutionary rate, low recombination and maternal inheritance (Elson & Lightowlers, 2006; Gissi et al., 2008). The mitochondrial genome can also provide genome-level characters, such as gene order, RNA secondary structures and conserved motif for replication and transcriptional control (Boore, 2006). These useful features can be utilized by comparative genomics for phylogenetic analysis, biological identification and population studies.

Leeches are clitellate annelids with the synapomorphies of a glandular clitellum, unique sperm morphology, hermaphroditism and direct development (Rouse & Fauchald, 1995). Due to the remarkable diversity in habitats that range from terrestrial to aquatic (both marine and freshwater) environments and important role for these ecosystems, leeches have been used as environmental stress indicators (Grantham & Hann, 1994). Nonsanguivorous leeches have been used as model organisms in neurobiological and developmental studies (Ferrier, 2012; Marrec-Croq et al., 2013). Additionally, the powerful anticoagulant (hirudin) in leech salivary secretions has been of interest to the field of medicine. Some species of leeches are also used in Traditional Chinese Medicine, including *Whitmania pigra*, *W. acranulata* and *Hirudo nipponia* (Zhang et al., 2013). The morphologies of *W. pigra* and *W. laevis* are similar, and the geographical ranges of *W. laevis*,

W. pigra and *W. acranulata* overlap broadly in central China (Tan, 2007). A clear phylogenetic framework and correct identification are helpful to the development and conservation of these diverse leeches. Existing information in GenBank regarding Hirudinea mitochondrial genomes is inadequate for phylogenetic studies of leeches and deep understanding of evolution and characteristics of the hirudinean mitochondrial genomes.

In this study, we present the complete and nearly complete mitochondrial genome sequences of *Whitmania laevis* and *Whitmania acranulata* respectively and describe both genome features. Then, we emphasize comparative analyses among all the complete mitochondrial genomes from *Whitmania* and highlight unique features and shared characteristics. Finally, we analyze phylogenetic relationships among Annelida.

MATERIALS AND METHODS

Specimen collection and DNA extraction

Specimens of *Whitmania laevis* (WLSX) and *W. acranulata* (WASX) were collected at Hanbin district (32°43'N, 108°46'E), Ankang, Shaanxi, China, and preserved in 95% ethanol at 4°C. DNA was extracted from the caudal sucker muscle tissue of single individuals using a TIANamp Micro DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol.

PCR and sequencing

Mitochondrial genomes of *W. laevis* (WLSX) and *W. acranulata* (WASX) were amplified with the primers listed in Table 1. PCR reactions were performed in a total volume of 25 µl, containing 2.5 mM $MgCl_2$, 2.5 µl 10 × LA PCR Buffer II (Mg^{2+} free), 0.4 mM of each dNTP, 1.25 U LA Taq polymerase, 0.4 µM of each primer, 45 ng gDNA. Cycling conditions were: an initial denaturation for 1 min at 93°C, followed by 40 cycles of 10 sec at 92°C, 30 sec at 46–57°C, 2–5 min at 68°C, and final extension of 10 min at 68°C. For nearly complete mitochondrial genome of *W. acranulata*, we were unable to amplify part of *ATP6* and *ND5* genes and the region between them with highly variable sequence and potential secondary structures. PCR products were purified with PCR Purification Kit (Sangon Biotech, Shanghai, China) and directly sequenced with the PCR primers and internal primers to complete sequences by primer walking.

Sequence analysis and Phylogenetic analyses

Contiguous sequence fragments were assembled using Staden Package v1.7.0 (Staden et al., 2000). Protein-coding and ribosomal RNA genes were initially identified using BLAST (Basic Local Alignment Search Tool) searches on GenBank, then by alignment with the published mitochondrial genome of *W. pigra* GenBank no. EU304459 (WP59). The secondary structure of the two rRNA genes was determined mainly by comparison with the published rRNA secondary structures of *Paragyrodactylus variegatus*, *Drosophila melanogaster* and *D. virilis* (Cannone et al., 2002; Ye et al., 2014). The program tRNAscan-SE v1.21 was used to identify tRNA genes and their potential cloverleaf structures (Lowe & Eddy, 1997). The tRNAs, which were not detected by tRNA scan-SE v1.21, were identified by comparison with *W. pigra*. The base composition and codon usage were calculated with MEGA v5.1 (Tamura et al., 2011). AT and GC skew were calculated according to the formulae: AT skew = (fA − fT) / (fA + fT) and GC skew = (fG − fC) / (fG + fC). To detect regions of highest variability, sliding window analyses were performed using DnaSP v5 (Librado & Rozas, 2009). A sliding window of 500 bp (in 25 bp overlapping steps) was used to estimate nucleotide diversity Pi (π) across the alignment of WLSX, WP59, *W. acranulata* GenBank no. KC688271 (WA71), *W. laevis* GenBank no. KC688269 (WL69), *Hirudo nipponia* GenBank no. KC667144 (HN44), *Hirudinaria manillensis* GenBank no.

TABLE 1

List of PCR primer combinations used to amplify the mitochondrial genomes of *Whitmania laevis* and *W. acranulata*.

Primer name	Sequence(5'-3')
Universal	
1F (rrnSF)	GGATTTAGTTGATGAACAACA
1R (ND1R)	CCTCAGCAAAATCAAATGG
2F (ND4F) [A]	TGRGGNTATCARCCNGARCG
2R (rrnSR)	CTACTATGTTACGACTTATCCT
3F (ND1F)	TGGCAGAGTAGTGCATTAGG
3R (COIR) [B]	GGTAATCAGAGTATCGWCGNGG
4F (COIF)	TGATTCTTTGGWCACCCWGAAGT
4R (COIIIR) [C]	ACWACGTCKACGAAGTGTCARTATCA
5F (CYTBF)	CAYATTAARCCWGARTGRTA
5R (ATP6R)	CCDGCHSTYATRTTDGCDGCWARHCG
6F (ND5F) [D]	ACNAAYCGWATYGGRGA
6F (ND5R) [D]	GCYTTAAATADHGCRTGDGT
Whitmania laevis	
WL1_COIIIF	AAAGATTTTGTGTATGC
WL1_TWR	TAACCTTTGA AGGGTTATAGTTT
WL2_ATP6F	TTAATAGTTGGACTTCCTCTCTGGG
WL2_ND5R	TGTCTATGGCATATCAATGACACTG
WL3_ND5F	CAACACCAGTGTCGGC
WL3_ND4R	CATTTTTGGGGCATGA
Whitmania acranulata	
WA1_COIIIF	ATTGCTGATAGGGTCTACGGT
WA1_CYTBR	ACACCCACCAATTCATGTTAA
WA2_ND5F	AGAGCTCAAATTCCATTC
WA2_ND4R	GGCTTTAGGCAACCATAG

Notes: A: Jennings & Halanych, 2005; B: Simon et al., 2006; C: Boore & Brown, 2000; D: Zhong et al., 2008.

KC688268 (HM68) and *Erpobdella octoculata* GenBank no. KC688270 (EO70) mitochondrial genomes. MrBayes ver.3.1.2 (Ronquist & Huelsenbeck, 2003) and RAxML ver.7.2.8 (Stamatakis et al., 2005) were used to draw a maximum likelihood (ML) and bayesian inference (BI) phylogeny based on part *COI* gene for leeches identification, and nine concatenated PCGs (*COI*, *COII*, *COIII*, *CYTB*, *ND1*, *ND2*, *ND3*, *ND4*, *ND5*) and two rRNA genes (Zhong et al., 2008) for phylogenetic relationships of Annelida. *Piscicola geometra*, and [*Terebratalia transversa and Laqueus rubellus*] were specified as the outgroups respectively. The best-fit model (GTR+Γ+I) for both datasets was estimated by ModelTest (Posada & Crandall, 1998). For ML analyses, bootstrap analysis was performed with 1,000 replicates. For BI analyses, two sets of four chains were allowed to run simultaneously for 1,000,000 generations. Each set was sampled every 100 generations with a burn-in of 25%. Stationarity was considered to be reached when the average standard deviation of split frequencies was less than 0.01.

RESULTS AND DISCUSSION

COI analysis of used species

COI gene is used as a standard DNA barcoding for many animal taxa. *COI* gene was also confirmed as a suitable marker for biological identification, and inter- and intraspecific relationships in leeches (Koperski et al., 2011; Kaygorodova & Mandzyak, 2014). To evaluate the validity of species used for comparative analyses of mitochondrial genomes, the *COI* phylogenetic

analysis based on all the relevant species data from GenBank was established. Both ML and BI trees showed a stable topology, which is similar to the findings of PHILLIPS & SIDDALL (2009), and major internal nodes were well-supported by bootstrap values and posterior probabilities (Fig. 1). All the representatives of *Hirudo nipponia*, *Hirudinaria manillensis* and *Erpobdella octoculata* are clustered together respectively, except for HN44, HM68 and EO70. These three last-listed specimens lie within the cluster formed by *Whitmania* species. This result suggests that these three individuals may have been erroneously identified. For the genus *Whitmania*, the different samples from *W. laevis* and *W. acranulata* are also not found in the same branches respectively. Thus, for comparative analyses of mitochondrial genomes, we employed all the *Whitmania* mitochondrial genome data from GenBank including HN44, HM68 and EO70.

Genome organization and base composition

The complete mitochondrial genome of *W. laevis* (WLSX) (GenBank no. KM655839) is 14,442 bp in length and contains 13 PCGs, 22 tRNA genes, and two rRNA genes (Fig. 2). The nearly complete mitochondrial genome of *W. acranulata* (WASX) (GenBank no. KM655838) has 13,494 bp, consisting of 13 PCGs, 20 tRNA genes, and two rRNA genes. The gene order of these genes in WLSX and WASX is identical to published *Whitmania* mitochondrial genomes, and all the genes are transcribed from the same strand in these leeches.

The overall A + T contents of WLSX and WASX are 73.0% and 72.4% respectively, which are similar to sequenced *Whitmania* spp. (Table 2). Statistically, nucleotide composition can be reflected by AT skew and GC skew (PERNA & KOCHER, 1995). The AT skew values

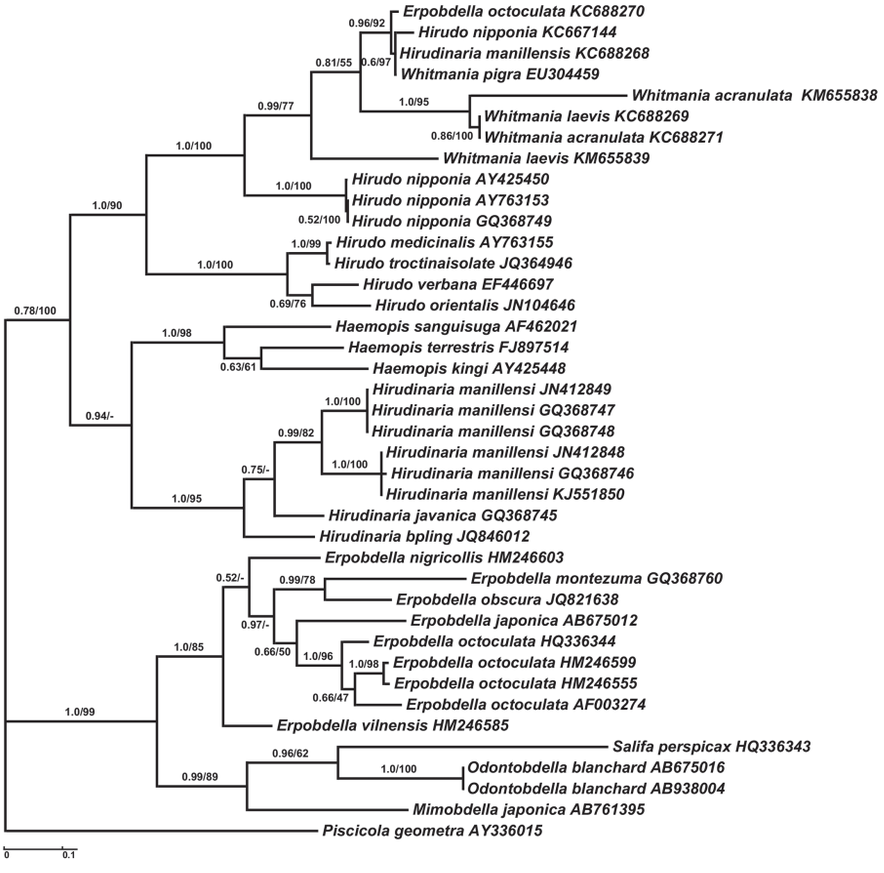

Fig. 1. – Phylogenetic reconstructions based on 40 *COI* gene sequences of leeches. First values at the branches correspond to Bayesian posterior probabilities while the second values indicate ML bootstrap support in percentages (ML bootstrap values < 50% are not shown).

TABLE 2

Nucleotide composition of *Whitmania* spp. mitochondrial genomes.

Feature	AT%							
	WLSX	WL69	WASX	WA71	WP59	HN44	HM68	EO70
Whole genome	73.0	71.9	72.4	71.6	72.2	72.6	72.0	71.6
Protein-coding genes	72.5	71.1	71.7	70.8	71.4	71.7	71.0	70.7
rrnL genes	73.5	73.2	73.6	74.1	73.0	74.5	75.1	73.0
rrnS genes	72.6	72.3	72.9	71.3	72.1	75.1	75.4	72.3
rRNA genes	73.1	72.8	73.3	73.0	72.7	74.7	75.2	72.7
tRNA genes	75.5	75.9	76.4	74.6	75.5	74.5	74.2	75.2
Feature	AT−skew							
	WLSX	WL69	WASX	WA71	WP59	HN44	HM68	EO70
Whole genome	-0.148	-0.144	-0.140	-0.140	-0.145	-0.127	-0.129	-0.135
Protein-coding genes	-0.192	-0.185	-0.182	-0.182	-0.191	-0.164	-0.168	-0.174
rrnL genes	-0.001	-0.002	-0.010	0.013	-0.001	-0.021	-0.010	0
rrnS genes	0.011	0.015	0.027	0.018	0.015	0.002	0.018	0.009
rRNA genes	0.004	0.004	0.004	0.015	0.005	-0.012	0.001	0.004
tRNA genes	-0.008	-0.034	0	-0.035	-0.012	0.002	-0.006	-0.021
Feature	GC−skew							
	WLSX	WL69	WASX	WA71	WP59	HN44	HM68	EO70
Whole genome	0.180	0.142	0.128	0.148	0.155	0.128	0.126	0.117
Protein-coding genes	0.168	0.123	0.109	0.140	0.144	0.117	0.108	0.095
rrnL genes	0.205	0.190	0.211	0.190	0.179	0.145	0.172	0.191
rrnS genes	0.228	0.216	0.168	0.206	0.216	0.217	0.198	0.222
rRNA genes	0.214	0.200	0.194	0.197	0.194	0.173	0.182	0.203
tRNA genes	0.232	0.211	0.204	0.177	0.215	0.197	0.199	0.191

Note: WLSX: *Whitmania laevis* KM655839, WL69: *Whitmania laevis* KC688269, WASX: *Whitmania acranulata* KM655838, WA71: *Whitmania acranulata* KC 688271, WP59: *Whitmania pigra* EU304459, HN44: *Hirudo nipponia* KC667144, HM68: *Hirudinaria manillensis* KC688268 and EO70: *Erpobdella octoculata* KC688270.

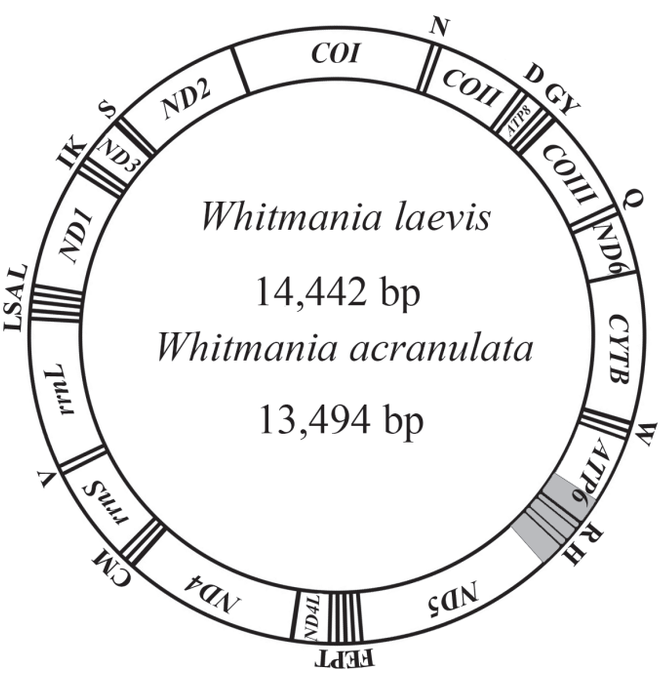

Fig. 2. – The gene map for the mitochondrial genome of *Whitmania laevis* and *W. acranulata*. The incomplete region of *W. acranulata* is in grey.

for the encoding strand of *Whitmania* spp. mitochondrial genomes are moderate T-skew, and GC skew values are moderate G-skew. These trends of AT and GC skew are also found in PCGs. In the rRNA genes, the bias of these leeches is moderate G-skew and weak A-skew, except for HN44 with weak T-skew (-0.012). The tRNAs show moderate G-skew and weak A-skew, except for HN44 (0.002, AT skew) and WASX (0, AT skew).

Protein-coding genes and codon usage

Four start codons are used in the PCGs of *Whitmania* mitochondrial genomes. GTG is found in all *COII* except for HN44 and WASX, in all *ND4L* except for HN44, WA71 and WASX, in all *ND5* except for WA71 and HM68, in all *ND1* except for WLSX and HM68, and in *ND3* for HN44, HM68, EO70 and WP59; TTG in all COIII, and the most frequent start codon is ATG in the other genes for *Whitmania* spp. In all *Whitmania* spp., five of 13 PCGs terminate with TAA (*ND5*, *ND4L*, *ATP6*, *ND3* and *CYTB*, expect for *ATP6* in HN44, *ND5* in HN44 and WP59); *ND2* terminate with incomplete-stop codons TA, and the remaining genes use the incomplete-stop codon T.

The average A + T content of PCGs for WLSX and WASX are 72.5% and 71.7%, respectively. It is similar to that of other *Whitmania* spp. (Table 2). This significant AT-richness is reflected in codon usage for mitochondrial proteins, which is similar to that observed in some other annelids (Boore, 2000; Zhong et al., 2008). In *Whitmania* mitochondrial genomes, all 64 codons in the mitochondrial genetic code table are used except for stop codon TAG in WLSX, WASX, WA71, HN44, HM68, EO70 and WP59. The most frequent amino acids in the PCGs are as follows: Leucine (15.06–15.96%), Serine (10.12–10.64%), Isoleucine (7.50–8.79%), Phenylalanine (8.00–8.60%), and Methionine (7.67–8.57%). UUA (Leucine), AUU (Isoleucine), UUU (Phenylalanine) and AUA (Methionine) are the most frequently used codons (Table 4).

Transfer RNA and ribosomal RNA genes

The length of large ribosomal subunit (*rrnL*) is 1,139 bp in WLSX and 1,133 bp in WASX, with an A + T content of 73.5% and 73.6%, respectively. The small ribosomal subunit (*rrnS*) is 736 bp in WLSX and 726 bp in WASX, and the A+T content is 72.6% and 72.9% for WLSX and WASX, respectively. The predicted secondary structure of *rrnL* and *rrnS* of WLSX is shown in Fig. 3 and Fig. 4, respectively. The secondary structure of *rrnL* contains six domains and 43 helices. But domain III is absent, which was reported in secondary structure of other invertebrate *rrnL* (Domes et al., 2008; Liu & Huang, 2010; Li et al., 2013). Among *Whitmania* spp. mitochondrial genomes, domains IV and V are more conserved than domains I, II, and VI. Overall, some helices (H235, H533, H589, H671, H687, H837, H946, H1057, H1196, H1648, H2023, H2347, H2675, and H2735) are greatly variable regions. The secondary structure of *rrnS* contains three domains and 27 helices. The domain III is more conserved than domains I and II. In domains I and II, conservative sites are mainly in helices H9, H367, H511, H769, H885 and loop of H673.

All of the 22 tRNA genes typical of metazoan mitochondrial genomes were identified in WLSX mitochondrial genome, while 20 tRNA genes were identified in WASX. All present tRNAs can be folded into the typical cloverleaf structure with the exception of $tRNA^{Pro}$ and $tRNA^{Gly}$ (Fig. 5). In $tRNA^{Pro}$ and $tRNA^{Gly}$, the TψC arm simply forms a loop. In addition, the TψC arm of other five tRNAs ($tRNA^{Ala}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Tyr}$ and $tRNA^{Val}$) is short with only one complementary base pair. The level of nucleotide conservation in tRNA genes is markedly different. The highest levels of nucleotide conservation occur in $tRNA^{Pro}$, $tRNA^{Leu(UUR)}$, $tRNA^{Asn}$ and $tRNA^{Met}$. However, $tRNA^{Arg}$, $tRNA^{His}$ and $tRNA^{Thr}$ show low levels of identical nucleotides among *Whitmania* spp.

TABLE 3

Annotation of the mitochondrial genomes of *Whitmania laevis* and *W. acranulata* (continued on next page).

Gene	From	To	Size (bp)	Start Codon	Stop Codon	Anticodon
Whitmania laevis						
COI	1	1534	1534	ATG	T	
tRNA-Asn (N)	1535	1596	62			GTT
COII	1597	2275	679	GTG	T	
tRNA-Asp (D)	2276	2339	64			GTC
ATP8	2340	2490	151	ATG	T	
tRNA-Gly (G)	2491	2549	59			TCC
tRNA-Tyr (Y)	2550	2610	61			GTA
COIII	2622	3402	781	TTG	T	
tRNA-Gln (Q)	3403	3471	69			TTG
ND6	3472	3928	457	ATG	T	
CYTB	3929	5074	1146	ATG	TAA	
tRNA-Trp (W)	5080	5140	61			TCA
ATP6	5204	5908	705	ATG	TAA	
tRNA-Arg (R)	5908	5970	63			TCG
tRNA-His (H)	6079	6139	61			GTG
ND5	6140	7835	1696	GTG	T	
tRNA-Phe (F)	7836	7897	62			GAA
tRNA-Glu (E)	7898	7958	61			TTC
tRNA-Pro (P)	7956	8016	61			TGG
tRNA-Thr (T)	8019	8078	60			TGT
ND4L	8079	8366	288	GTG	TAA	
ND4	8360	9692	1333	ATG	T	
tRNA-Cys (C)	9702	9762	61			GCA
tRNA-Met (M)	9763	9825	63			CAT
rrnS (12S)	9826	10561	736			
tRNA-Val (V)	10562	10623	62			TAC
rrnL(16S)	10624	11762	1139			
tRNA-Leu$^{(CUN)}$(L1)	11763	11823	61			TAG
tRNA-Ser$^{(UCN)}$(S2)	11823	11890	68			TGA
tRNA-Ala (A)	11891	11950	60			TGC
tRNA-Leu$^{(UUR)}$(L2)	11951	12011	61			TAA
ND1	12012	12930	919	ATG	T	
tRNA-Ile (I)	12931	12992	62			GAT
tRNA-Lys (K)	12994	13055	62			TTT
ND3	13057	13401	345	ATG	TAA	
tRNA-Ser$^{(AGN)}$(S1)	13388	13454	67			TCT
ND2	13455	14437	983	ATG	TA	
Whitmania acranulata						
ND5	1	1260	1260		TAA	
tRNA-Phe (F)	1260	1321	62			GAA
tRNA-Glu (E)	1322	1380	59			TTC
tRNA-Pro (P)	1378	1436	59			TGG
tRNA-Thr (T)	1438	1497	60			TGT
ND4L	1498	1785	288	ATG	TAA	
ND4	1779	3111	1333	ATG	T	
tRNA-Cys (C)	3121	3181	61			GCA
tRNA-Met (M)	3182	3243	62			CAT
rrnS (12S)	3244	3969	726			
tRNA-Val (V)	3970	4035	66			TAC
rrnL(16S)	4036	5168	1133			
tRNA-Leu$^{(CUN)}$(L1)	5172	5231	60			TAG
tRNA-Ser$^{(UCN)}$(S2)	5231	5298	68			TGA
tRNA-Ala (A)	5299	5358	60			TGC
tRNA-Leu$^{(UUR)}$(L2)	5359	5419	61			TAA

Gene	From	To	Size (bp)	Start Codon	Stop Codon	Anticodon
ND1	5420	6338	919	GTG	T	
tRNA-Ile (I)	6339	6400	62			GAT
tRNA-Lys (K)	6401	6462	62			TTT
ND3	6464	6808	345	ATG	TAA	
tRNA-Ser$^{(AGN)}$(S1)	6795	6861	67			TCT
ND2	6862	7844	983	ATG	TA	
COI	7850	9383	1534	ATG	T	
tRNA-Asn (N)	9384	9445	62			GTT
COII	9446	10127	682	ATG	T	
tRNA-Asp (D)	10128	10193	66			GTC
ATP8	10194	10341	148	ATG	T	
tRNA-Gly (G)	10345	10404	60			TCC
tRNA-Tyr (Y)	10405	10464	60			GTA
COIII	10450	11245	796	TTG	T	
tRNA-Gln (Q)	11246	11314	69			TTG
ND6	11314	11775	462	ATG	T	
CYTB	11776	12921	1146	ATG	TAA	
tRNA-Trp (W)	12925	12984	60			TCA
ATP6	13040	13494	455	ATG		

Non-coding regions

Whitmania spp. mitochondrial genomes are highly compacted in genome size as in other animals (Boore, 1999). A total of 7 short non-coding regions were identified ranging from 1 bp to 11 bp in the mitochondrial genome of WLSX (Table 3). There are two major non-coding regions (NCR1 and NCR2) in the same positions of HN44, WL69 and WP59 mitochondrial genome, while the remaining ones have one non-coding region (NCR2). NCR1 and NCR2 are located between *tRNA*^{Trp} and *ATP6*, and *tRNA*^{Arg} and *tRNA*^{His}, respectively. The NCR1 and NCR2 are too variable for alignments, but the sequence similarity of NCR1 between WLSX and WP59 is 63.4% and the NCR2 has 53.2% sequence similarity. The NCR1 contains two stem-loop

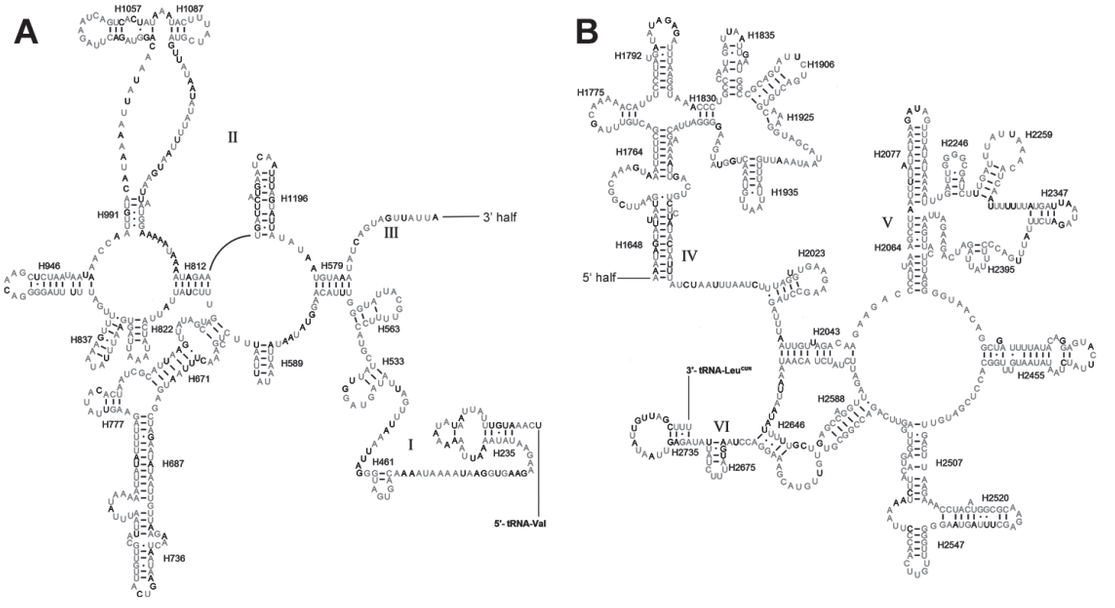

Fig. 3. – Inferred secondary structure of the mitochondrial *rrnL* gene for *Whitmania laevis*. Conserved nucleotides of all *Whitmania* taxa are labelled in grey.

TABLE 4

Codon usage for *Whitmania* spp. mitochondrial protein coding genes.

Codon (AA)	Number of used codon								Relative synonymous codon usage							
	WLSX	WL69	WASX	WA71	WP59	HN44	HM68	EO70	WLSX	WL69	WASX	WA71	WP59	HN44	HM68	EO70
UUU(F)	277	263	264	262	265	279	257	257	1.80	1.70	1.81	1.75	1.75	1.79	1.75	1.63
UUC(F)	31	46	28	37	38	32	37	58	0.20	0.30	0.19	0.25	0.25	0.21	0.25	0.37
UUA(L)	382	351	326	341	357	348	341	322	4.04	3.69	3.72	3.65	3.67	3.80	3.60	3.46
UUG(L)	84	91	71	92	101	73	97	92	0.89	0.96	0.81	0.98	1.04	0.80	1.02	0.99
CUU(L)	47	52	38	51	50	45	48	58	0.50	0.55	0.43	0.55	0.51	0.49	0.51	0.62
CUC(L)	4	10	6	11	11	12	16	13	0.04	0.11	0.07	0.12	0.11	0.13	0.17	0.14
CUA(L)	40	57	71	52	55	61	55	61	0.42	0.60	0.81	0.56	0.57	0.67	0.58	0.65
CUG(L)	10	10	14	14	9	10	11	13	0.11	0.11	0.16	0.15	0.09	0.11	0.12	0.14
AUU(I)	292	264	263	243	270	261	268	265	1.82	1.75	1.76	1.77	1.75	1.76	1.75	1.74
AUC(I)	29	37	36	31	38	35	38	39	0.18	0.25	0.24	0.23	0.25	0.24	0.25	0.26
AUA(I)	235	211	232	220	223	230	226	216	1.54	1.47	1.57	1.47	1.50	1.53	1.52	1.54
AUG(M)	71	76	63	79	74	70	72	65	0.46	0.53	0.43	0.53	0.50	0.47	0.48	0.46
GUU(V)	108	112	97	123	115	107	107	113	1.53	1.59	1.52	1.66	1.53	1.60	1.52	1.59
GUC(V)	16	17	16	15	17	13	17	18	0.23	0.24	0.25	0.20	0.23	0.19	0.24	0.25
GUA(V)	119	122	111	129	133	120	131	122	1.69	1.74	1.74	1.74	1.77	1.80	1.86	1.71
GUG(V)	39	30	31	30	35	27	27	32	0.55	0.43	0.49	0.40	0.47	0.40	0.38	0.45
UCU(S)	99	95	103	106	99	98	95	87	2.06	1.98	2.28	2.18	2.12	2.08	2.04	1.83
UCC(S)	21	25	14	18	19	20	21	24	0.44	0.52	0.31	0.37	0.41	0.42	0.45	0.51
UCA(S)	101	90	85	88	95	93	93	105	2.10	1.88	1.88	1.81	2.04	1.97	2.00	2.21
UCG(S)	17	19	17	22	19	17	17	20	0.35	0.40	0.38	0.45	0.41	0.36	0.37	0.42
CCU(P)	55	56	58	51	50	53	55	49	1.63	1.49	1.72	1.46	1.45	1.54	1.44	1.35
CCC(P)	3	10	12	10	11	4	17	12	0.09	0.27	0.36	0.29	0.32	0.12	0.44	0.33
CCA(P)	66	66	58	61	59	64	64	68	1.96	1.76	1.72	1.74	1.71	1.86	1.67	1.88
CCG(P)	11	18	7	18	18	17	17	16	0.33	0.48	0.21	0.51	0.52	0.49	0.44	0.44
ACU(T)	64	62	70	68	69	76	68	71	1.97	1.92	2.15	1.99	2.08	2.01	1.94	1.97
ACC(T)	9	15	7	14	11	9	17	14	0.28	0.47	0.22	0.41	0.33	0.24	0.49	0.39
ACA(T)	46	45	46	43	43	55	45	49	1.42	1.40	1.42	1.26	1.29	1.46	1.29	1.36
ACG(T)	11	7	7	12	10	11	10	10	0.34	0.22	0.22	0.35	0.30	0.29	0.29	0.28
GCU(A)	71	60	71	68	61	64	69	62	2.06	1.85	2.15	1.88	1.82	1.91	1.94	1.81
GCC(A)	9	15	24	20	22	16	22	23	0.26	0.46	0.73	0.55	0.66	0.48	0.62	0.67
GCA(A)	50	46	30	47	43	47	42	44	1.45	1.42	0.91	1.30	1.28	1.40	1.18	1.28
GCG(A)	8	9	7	10	8	7	9	8	0.23	0.28	0.21	0.28	0.24	0.21	0.25	0.23
UAU(Y)	148	148	133	143	128	133	132	135	1.74	1.67	1.56	1.59	1.57	1.59	1.58	1.59
UAC(Y)	22	29	37	37	35	34	35	35	0.26	0.33	0.44	0.41	0.43	0.41	0.42	0.41
UAA(*)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UAG(*)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CAU(H)	60	67	55	64	61	53	59	59	1.82	1.94	1.69	1.86	1.91	1.71	1.79	1.79
CAC(H)	6	2	10	5	3	9	7	7	0.18	0.06	0.31	0.14	0.09	0.29	0.21	0.21
CAA(Q)	33	41	38	40	35	42	44	43	1.22	1.46	1.49	1.38	1.32	1.42	1.49	1.46
CAG(Q)	21	15	13	18	18	17	15	16	0.78	0.54	0.51	0.62	0.68	0.58	0.51	0.54
AAU(N)	120	120	108	119	120	128	125	113	1.78	1.74	1.73	1.76	1.75	1.74	1.72	1.67
AAC(N)	15	18	17	16	17	19	20	22	0.22	0.26	0.27	0.24	0.25	0.26	0.28	0.33
AAA(N)	66	78	66	75	74	82	81	88	1.28	1.46	1.42	1.47	1.42	1.50	1.42	1.54
AAG(K)	37	29	27	27	30	27	33	26	0.72	0.54	0.58	0.53	0.58	0.50	0.58	0.46
GAU(D)	72	65	61	62	78	81	68	70	1.71	1.69	1.63	1.61	1.73	1.71	1.64	1.59
GAC(D)	12	12	14	15	12	14	15	18	0.29	0.31	0.37	0.39	0.27	0.29	0.36	0.41
GAA(E)	41	43	39	49	46	48	55	50	1.14	1.21	1.15	1.18	1.30	1.23	1.39	1.32
GAG(E)	31	28	29	34	25	30	24	26	0.86	0.79	0.85	0.82	0.70	0.77	0.61	0.68
UGU(C)	55	56	44	52	50	42	48	45	1.83	1.65	1.66	1.65	1.61	1.40	1.60	1.53
UGC(C)	5	12	9	11	12	18	12	14	0.17	0.35	0.34	0.35	0.39	0.60	0.40	0.47
UGA(W)	77	74	66	76	74	78	76	79	1.56	1.45	1.38	1.42	1.53	1.58	1.50	1.52
UGG(W)	22	28	30	31	23	21	25	25	0.44	0.55	0.63	0.58	0.47	0.42	0.50	0.48
CGU(R)	18	17	13	16	20	21	17	18	1.33	1.13	1.08	1.08	1.43	1.47	1.15	1.29
CGC(R)	2	5	3	2	4	5	4	5	0.15	0.33	0.25	0.14	0.29	0.35	0.27	0.36
CGA(R)	25	28	26	33	26	26	30	28	1.85	1.87	2.17	2.24	1.86	1.82	2.03	2.00
CGG(R)	9	10	6	8	6	5	8	5	0.67	0.67	0.50	0.54	0.43	0.35	0.54	0.36
AGU(S)	42	48	37	49	48	46	47	48	0.88	1.00	0.82	1.01	1.03	0.98	1.01	1.01
AGC(S)	8	9	9	8	4	9	7	5	0.17	0.19	0.20	0.16	0.09	0.19	0.15	0.11
AGA(S)	60	71	64	68	60	64	64	59	1.25	1.48	1.42	1.40	1.29	1.36	1.38	1.24
AGG(S)	36	26	32	30	29	30	28	32	0.75	0.54	0.71	0.62	0.62	0.64	0.60	0.67
GGU(G)	77	61	68	63	71	70	73	63	1.66	1.27	1.57	1.39	1.51	1.47	1.57	1.36
GGC(G)	16	26	18	17	15	20	13	19	0.34	0.54	0.42	0.38	0.32	0.42	0.28	0.41
GGA(G)	41	48	34	49	42	54	46	44	0.88	1.00	0.79	1.08	0.89	1.14	0.99	0.95
GGG(G)	52	57	53	52	60	46	54	59	1.12	1.19	1.23	1.15	1.28	0.97	1.16	1.28

Notes: WLSX: *Whitmania laevis* KM655839, WL69: *Whitmania laevis* KC688269, WASX: *Whitmania acranulata* KM655838, WA71: *Whitmania acranulata* KC 688271, WP59: *Whitmania pigra* EU304459, HN44: *Hirudo nipponia* KC667144, HM68: *Hirudinaria manillensis* KC688268, EO70: *Erpobdella octoculata* KC688270 and AA: amino acid.

structures at positions 4-21 bp and 27-45 bp in WLSX. Two stem-loop structures were also found in NCR2. The conserved sequences of both NCR1 and NCR2 between WLSX and WP59 mainly occur in the stem-loop structures. Tandem repeat sequences commonly observed in other invertebrate lineages (ZHANG & HEWITT, 1997) were not found in NCR1 and NCR2 for *Whitmania* mitochondrial genomes.

Sliding window analyses and nucleotide diversity

Sliding window analysis was performed to estimate nucleotide diversity Pi (π) for the mitochondrial genome of *Whitmania*. Not unexpectedly, the most variable regions were found in the major non-coding regions (Fig. 6). The sliding window indicated that the most

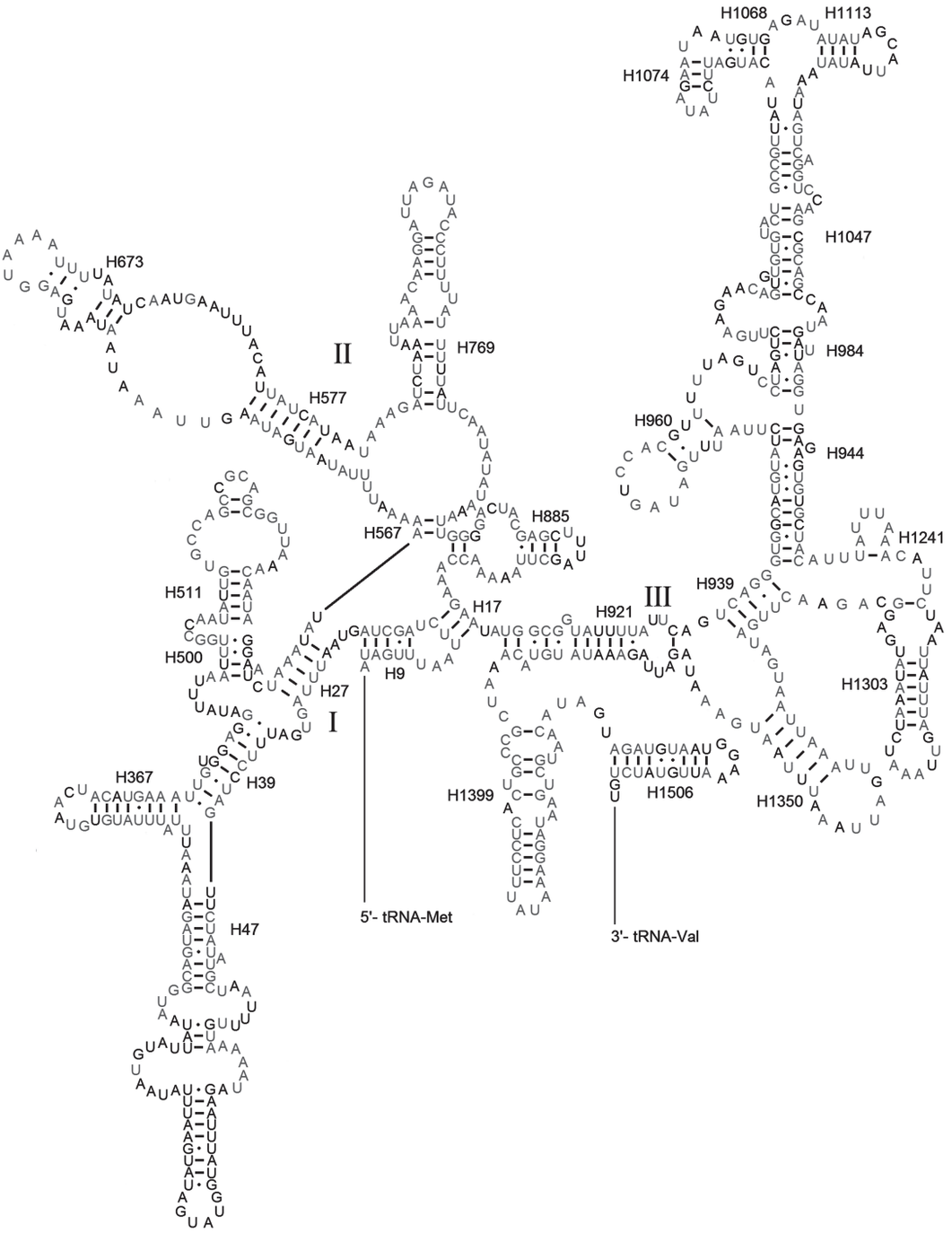

Fig. 4. – Inferred secondary structure of the mitochondrial *rrnS* gene for *Whitmania laevis*. Conserved nucleotides of all *Whitmania* taxa are labelled in grey.

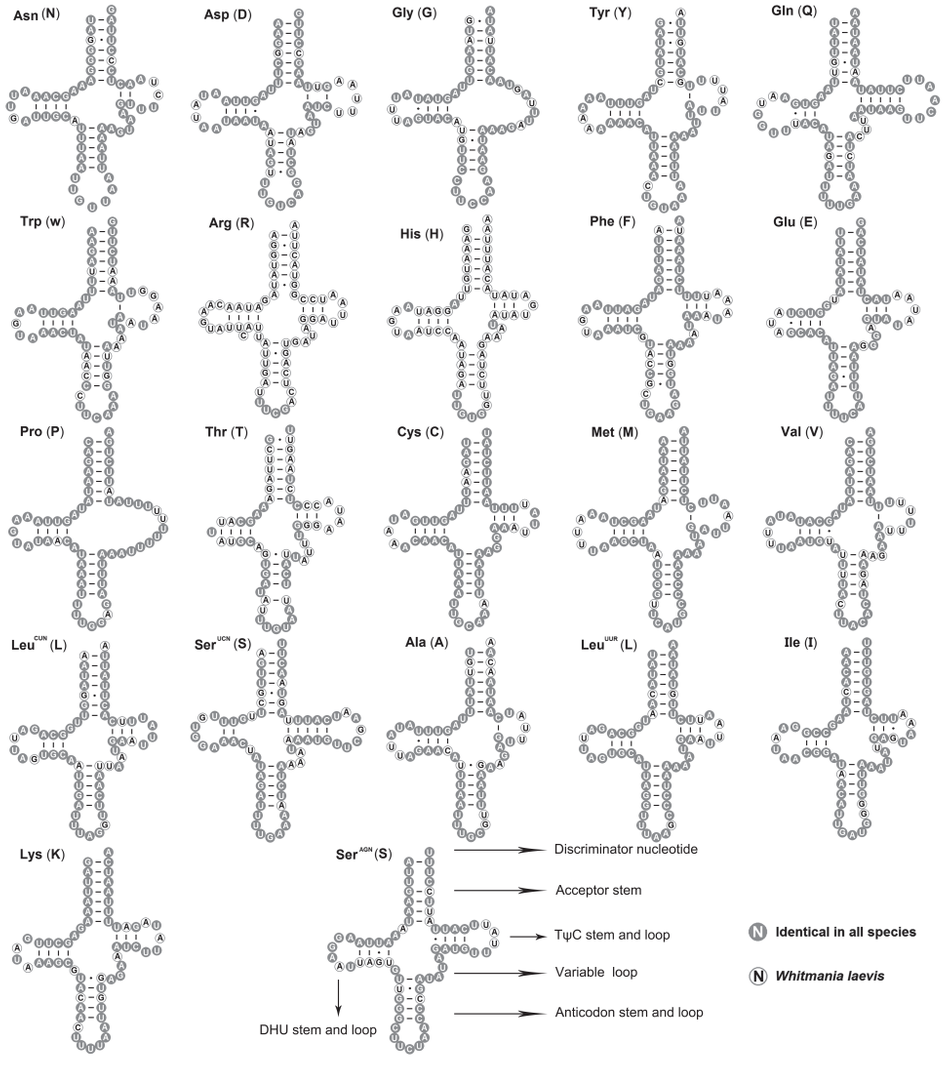

Fig. 5. – The inferred secondary structures of mitochondrial tRNA genes of *Whitmania laevis.*

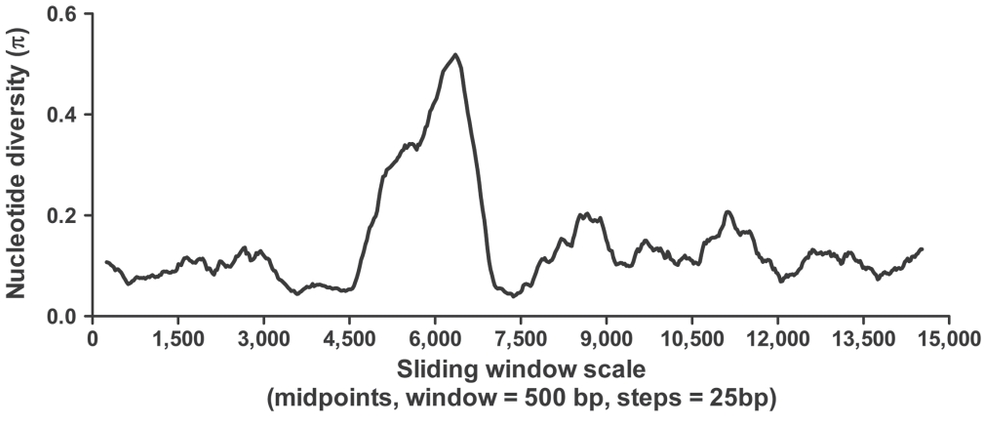

Fig. 6. – Sliding window analyses of the alignment among *Whitmania* spp. mitochondrial genomes. The line shows the value of nucleotide diversity (π) in a sliding window analysis of window size 500 bp with step size 25; the value is inserted at its mid-point.

variable coding regions were within the genes *ATP6* and 5' part of *ND5* (Fig. 6). Amongst PCGs the most conserved gene fragments are the 3' end of *COIII*, *ND6* and 5' part of *CYTB*. By contrast, the most variable regions in *ATP6*, *ND5* and *ND4* genes can be used as effective markers to investigate relationships of populations and the closely related species.

Phylogenetic analyses

Annelida, the segmented worms, traditionally includes two taxonomic groups, namely clitellates and polychaetes. Recently, analyses of molecular data indicate Annelida may contain several other phyla (Struck et al., 2007; Zrzavý et al., 2009), but the evolution and phylogeny of Annelida is still controversial. In Euhirudinea, although the relationships within Hirudiniformes have been extensively investigated (Apakupakul et al., 1999; Borda & Siddall, 2004; Borda et al., 2008; Phillips & Siddall, 2009), few relationships of closely related species within *Whitmania* have as yet been clearly elucidated. In order to infer phylogenetic relationships of annelids, especially for these closely related species within *Whitmania*, the nucleotide dataset of concatenated nine PCGs and two rRNA genes were employed for phylogenetic analysis. Both ML and BI analysis showed similar tree topologies (Fig. 7). The results of the *Whitmania* branch revealed that *W. laevis* and *W. pigra* were closely related with high statistical support without considering the uncertain species HN44, HM68, EO70. Our results of *Whitmania* (*W. acranulata* + (*W. laevis* + *W. pigra*)) differ from the results of Xu et al. (2013) based on only three mitochondrial genes. Compared with reported molecular phylogenies (Rousset et al., 2007; Struck et al., 2007; Shen et al., 2009), Clitellata appears consistently as a monophyletic group; Sipunculans form a sister group of annelids (including echiurans); *Clymenella torquata* (Capitellida) clusters with two Terebellida species. With greater numbers of species in mitochodrial genomic analyses, the phylogenetic positions of Echiurida and some groups within

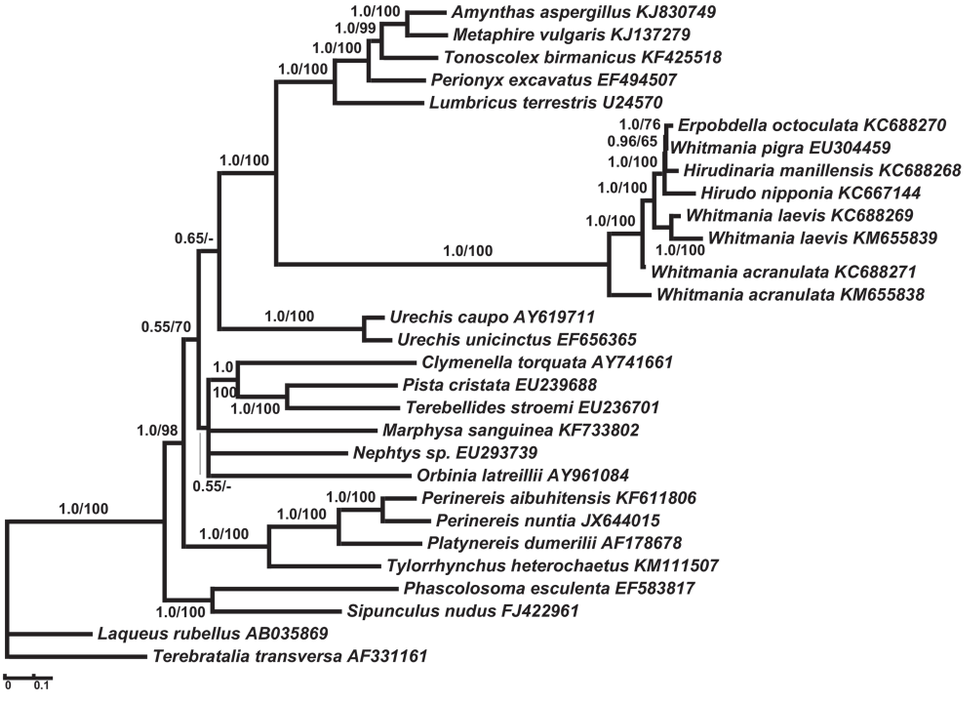

Fig. 7. – Phylogenetic tree inferred from nine PCGs and two rRNA genes using BI and ML analysis. First values at the branches correspond to Bayesian posterior probabilities while the second values indicate ML bootstrap support in percentages (ML bootstrap values < 50% are not shown).

Polychaeta appear quite different (ZHONG et al., 2008; SHEN et al., 2011). The Echiurida and Clitellata cluster together as a sister clade and the branch consists of the cluster Maldanidae/ Terebellida, *Marphysa sanguinea* (Eunicidae), *Orbinia latreillii* (Orbiniidae) and *Nephtys* sp. (Nephtyidae) with low nodal support suggesting that their relationships still need to be investigated with a broader taxonomic sample. Furthermore, differing topologies derived from nuclear and mitochondrial data sets indicate the need for more investigation of the "symplesiomorphy trap" in Annelida (ZHONG et al., 2011).

CONCLUSIONS

The mitochondrial genomes of *W. laevis* and *W. acranulata* display identical genome organization and gene order to previously reported *Whitmania* mitochondrial genomes. Comparative analyses of *Whitmania* mitochondrial genomes reveal: (i) the nucleotide composition is significantly biased toward A and T; (ii) the significant AT-richness is reflected in codon usage with frequent UUA, AUU, UUU, and AUA; (iii) the TψC arm of five tRNAs ($tRNA^{Ala}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Tyr}$ and $tRNA^{Val}$) is short with only one complementary base pair; (iv) domain III in rrnS and domains IV and V in rrnL are the most conserved parts. The sliding window analysis reveals that *ND4*, *ND5* and *ATP6* genes may serve as useful markers to investigate relationships of population and of closely related species. The phylogenetic analysis based on nine PCGs and two rRNA genes confirms *W. laevis* and *W. pigra* are closely related with high statistical support. The comparative analyses of *Whitmania* mitochondrial genomes could provide more information for understanding of the characteristics and evolution of the *Whitmania* mitochondrial genomes.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Yuan Huang (College of Life Science, Shaanxi Normal University) for assistance with the phylogenetic analyses and Dr Zhongmin Dong (Department of Biology, Saint Mary's University) for editorial comments on an early draft of the manuscript. This research was supported by the National Natural Science Foundation of China (31372158).

REFERENCES

APAKUPAKUL K, SIDDALL ME & BURRESON EM (1999). Higher level relationships of leeches (Annelida: Clitellata: Euhirudinea) based on morphology and gene sequences. Molecular Phylogenetics and Evolution, 12: 350–359.

BOORE JL (1999). Animal mitochondrial genomes. Nucleic Acids Research, 27: 1767–1780.

BOORE JL (2006). The use of genome-level characters for phylogenetic reconstruction. Trends in Ecology and Evolution, 21: 39–446.

BOORE JL & BROWN WM (2000). Mitochondrial genomes of Galathealinum, Helobdella, and Platynereis: Sequence and gene arrangement comparisons indicate that Pogonophora is not a phylum and Annelida and Arthropoda are not sister taxa. Molecular Biology and Evolution, 17: 87–106.

BORDA E, OCEGUERA-FIGUEROA A & SIDDALL ME (2008). On the classification, evolution and biogeography of terrestrial haemadipsoid leeches (Hirudinida: Arhynchobdellida: Hirudiniformes). Molecular Phylogenetics and Evolution, 46: 142–154.

BORDA E & SIDDALL ME (2004). Arhynchobdellida (Annelida: Oligochaeta: Hirudinida): phylogenetic relationships and evolution. Molecular Phylogenetics and Evolution, 30: 213–225.

CANNONE JJ, SUBRAMANIAN S, SCHNARE MN, COLLETT JR, D'SOUZA LM, DU Y, FENG B, LIN N, MADABUSI LV, MÜLLER KM, PANDE N, SHANG Z, YU N & GUTELL RR (2002). The Comparative RNA Web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BMC Bioinformatics, 3: 2.

DOMES K, MARAUN M, SCHEU S & CAMERON SL (2008). The complete mitochondrial genome of the sexual oribatid mite *Steganacarus magnus*: genome rearrangements and loss of tRNAs. BMC Genomics, 9: 532.

Elson JL & Lightowlers RN (2006). Mitochondrial DNA clonality in the dock: can surveillance swing the case? Trends in Genetics, 22: 603–607.

Ferrier DE (2012). Evolutionary crossroads in developmental biology: annelids. Development, 139: 2643–2653.

Gissi C, Iannelli F & Pesole G (2008). Evolution of the mitochondrial genome of Metazoa as exemplified by comparison of congeneric species. Heredity, 101: 301–320.

Grantham BA & Hann BJ (1994). Leeches (Annelida: Hirudinea) in the experimental lakes area, Northwestern Ontario, Canada: Patterns of species composition in relation to environment. Canadian Journal of Fisheries and Aquatic Sciences, 51: 1600–1607.

Jennings RM & Halanych KM (2005). Mitochondial genomes of *Clymenella torquata* (Maldanidae) and *Rifta pachyprila* (Siboglinidae): evidence for conserved gene order in Annelida. Molecular Biology and Evolution, 22: 210–222.

Kaygorodova IA & Mandzyak NB (2014). Molecular phylogeny of siberian Glossiphoniidae (Hirudinea). Molecular Biology, 48: 452–455.

Koperski P, Milanowski R & Krzyk A (2011). Searching for cryptic species in *Erpobdella octoculata* (L.) (Hirudinea: Clitellata): discordance between the results of genetic analysis and cross-breeding experiments. Contributions to Zoology, 80: 85–94.

Li T, Gao C, Cui Y, Xie Q & Bu W (2013). The Complete Mitochondrial Genome of the Stalk-Eyed Bug *Chauliops fallax* Scott, and the Monophyly of Malcidae (Hemiptera: Heteroptera). PLoS ONE, 8: e55381.

Librado P & Rozas J (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25: 1451–1452.

Liu N & Huang Y (2010). Complete mitochondrial genome sequence of *Acrida cinerea* (Acrididae: Orthoptera) and comparative analysis of mitochondrial genomes in Orthoptera. Comparative and Functional Genomics, 2010: 319486.

Lowe TM & Eddy SR (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Research, 25: 955–964.

Marrec-Croq FL, Drago F, Vizioli J, Sautière PE & Lefebvre C (2013). The leech nervous system: A valuable model to study the microglia involvement in regenerative processes. Clinical and Developmental Immunology, 2013: 274019.

Perna NT & Kocher TD (1995). Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. Journal of Molecular Evolution, 41: 353–358.

Phillips AJ & Siddall ME (2009). Poly-paraphyly of Hirudinidae: many lineages of medicinal leeches. BMC Evolutionary Biology, 9: 246.

Posada D & Crandall KA (1998). Modeltest: Testing the model of DNA substitution. Bioinformatics, 14: 817–818.

Ronquist F & Huelsenbeck JP (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19: 1572–1574.

Rouse GW & Fauchald K (1995). The articulation of annelids. Zoologica Scripta, 4: 269–301.

Rousset V, Pleijel F, Rouse GW, Erseus C & Siddali ME (2007). A molecular phylogeny of annelids. Cladistics, 23: 41–63.

Sakai M & Sakaizumi M (2012). The complete mitochondrial genome of *Dugesia japonica* (Platyhelminthes; Order Tricladida). Zoological Science, 29: 672–680.

Shen X, Ma X, Ren J & Zhao F (2009). A close phylogenetic relationship between Sipuncula and Annelida evidenced from the complete mitochondrial genome sequence of *Phascosoma esculenta*. BMC Genomics, 10: 136.

Shen X, Wu Z, Sun M, Ren J & Liu B (2011). The complete mitochondrial genome sequence of *Whitmania pigra* (Annelida, Hirudinea): The first representative from the class Hirudinea. Comparative Biochemistry and Physiology D, 6: 133–138.

Simon C, Buckley TR, Frati F, Stewart JB & Beckenbach AT (2006). Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. Annual Review of Ecology Evolution and Systematics, 37: 545–579.

Staden R, Beal KF & Bonfield JK (2000). The Staden package, 1998. Methods in Molecular Biology, 132: 115–130.

Stamatakis A, Ludwig T & Meier H (2005). RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics, 21: 456–463.

Struck TH, Schult N, Kusen T, Hickman E, Bleidorn C, McHugh D & Halanych KM (2007). Annelid phylogeny and the status of Sipuncula and Echiura. BMC Evolutionary Biology, 7: 57.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M & Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731–2739.

Tan N (2007). Zoogeoraphy of Hirudinidae in China. Acta Scientiarum Naturalium Universitatis Sunyatseni, 46: 100–104.

Xu Y, Nie J & Xiao L (2013). Molecular evolution analysis of COI, 12S rRNA and 16S rRNA gene in six species of leech. Journal of Biology, 30: 10–13.

Ye F, King SD, Cone DK & You P (2014). The mitochondrial genome of *Paragyrodactylus variegatus* (Platyhelminthes: Monogenea): differences in major non-coding region and gene order compared to *Gyrodactylus*. Parasites & Vectors, 7: 377.

Zhong M, Struck TH & Halanych KM (2008). Phylogenetic information from three mitochondrial genomes of Terebelliformia (Annelida) worms and duplication of the methionine tRNA. Gene, 416: 11–21.

Zhong M, Hansen B, Nesnidal M, Golombek A, Halanych KM & Struck TH (2011). Detecting the symplesiomorphy trap: a multigene phylogenetic analysis of terebelliform annelids. BMC Evolutionary Biology, 11: 369.

Zhang W, Zhang R, Li J, Liang F & Qian Z (2013). Species study on Chinese medicine leech and discussion on its resource sustainable utilization. China Journal of Chinese material medica, 38: 914–918.

Zhang DX & Hewitt GM (1997). Insect mitochondrial control region: a review of its structure, evolution and usefulness in evolutionary studies. Biochemical Systematics and Ecology, 25: 99–120.

Zrzavý J, Říha P, Piálek L & Janouškovec J (2009). Phylogeny of Annelida (Lophotrochozoa): total-evidence analysis of morphology and six genes. BMC Evolutionary Biology, 9: 189.

Received: September 29th, 2014

Accepted: March 24th, 2015

Branch editor: Kurt Jordaens

Belg. J. Zool., 145(2) : 130-136 July 2015

SHORT NOTE

First records of *Myotis alcathoe* von Helversen & Heller, 2001 in Belgium

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KEYWORDS: Vespertilionidae, Alcathoe whiskered bat, Wallonia, faecal DNA extraction.

Molecular techniques have led to the discovery of several cryptic bat taxa in Europe (1, 2). One of these recently-discovered species is *Myotis alcathoe* von Helversen & Heller, 2001, a species in the 'whiskered bat'-complex (3). *M. alcathoe* is morphologically very similar to the whiskered bat *M. mystacinus* (Kuhl, 1817) and to the Brandt's bat *M. brandtii* (Eversmann, 1845), even though it is not a sister-taxon to either of these species (4).

Although molecular techniques remain the most useful and reliable identification method (5), the species can also be identified based on a number of morphological characteristics, most notably its very small size (forearm < 33.5 mm) and the well-developed protoconus of the third upper premolar (3, 6). Other distinctive traits for the species are the pink face, shape of the penis, short tragus and short snout (6).

M. alcathoe is regarded as a forest specialist, and is most often observed in moist and old growth deciduous forest during summer (7, 8). Summer roost sites are generally situated in tree cavities, and are difficult to find (8). As do many other vespertilionid bat species, *M. alcathoe* visits caves and similar underground sites in autumn to swarm, a behaviour linked with mating (9). Very little is known about the hibernation behaviour of the species. As species identification often requires handling – and thus disturbance – of the bat, species of the 'whiskered bat'-complex are most often not identified to the species level during winter surveys. However, *M. alcathoe* has been recorded at caves during winter (e.g. 10, 11).

The species was originally described from specimens from Greece (3), but soon found to have a widespread – but patchy – distribution in a large part of Europe (7). In northwest Europe, *M. alcathoe* has been recorded in Germany (7), France (7, 12), the UK (13) and the Grand Duchy of Luxembourg (14). There have also been observations close to the Belgian border in Luxembourg and France (departments Pas de Calais, Ardennes and Meuse), and its presence in Belgium could thus be expected (7). In this short note, we present the first records of *M. alcathoe* in Belgium.

Table 1

Measurements, age (based on the ossification of epiphyseal joints) and sex of *Myotis alcathoe* caught in Belgium. FA = forearm length. Individuals used for genetic analyses are indicated with ** (cyt b and ND1) and * (cyt b).

Capture date	Site	Age	Sex	Sexual status	Mass (g)	FA (mm)	
11/07/11	Bois de Saint-Rémy	Adult	F	Post lactating	5	32	
30/07/11	Bois de Saint-Rémy	Adult	M		5	31,9	
04/10/11	Grotte touristique de Rochefort	Adult	F	non lactating	4	33,5	
04/10/11	Grotte touristique de Rochefort	Adult	F	non lactating	5,5	32,6	
04/10/11	Grotte touristique de Rochefort	Adult	F	non lactating	5,5	33,1	
04/10/11	Grotte touristique de Rochefort	Adult	F	non lactating	6	32,4	
25/09/12	Grotte touristique de Rochefort	Juvenile	M		5	31,5	
12/10/12	Grotte touristique de Rochefort	Adult	M		4	31,6	
12/10/12	Grotte touristique de Rochefort	Adult	M		5	32,7	
15/08/14	Etang des Prés de Virelles	Adult	M		5	33,1	
02/09/14	Grotte touristique de Rochefort	Juvenile	M		4,5	32,8	**
02/09/14	Grotte touristique de Rochefort	Juvenile	M		4,6	32,5	*
02/09/14	Grotte touristique de Rochefort	Juvenile	F		4,3	32,4	*

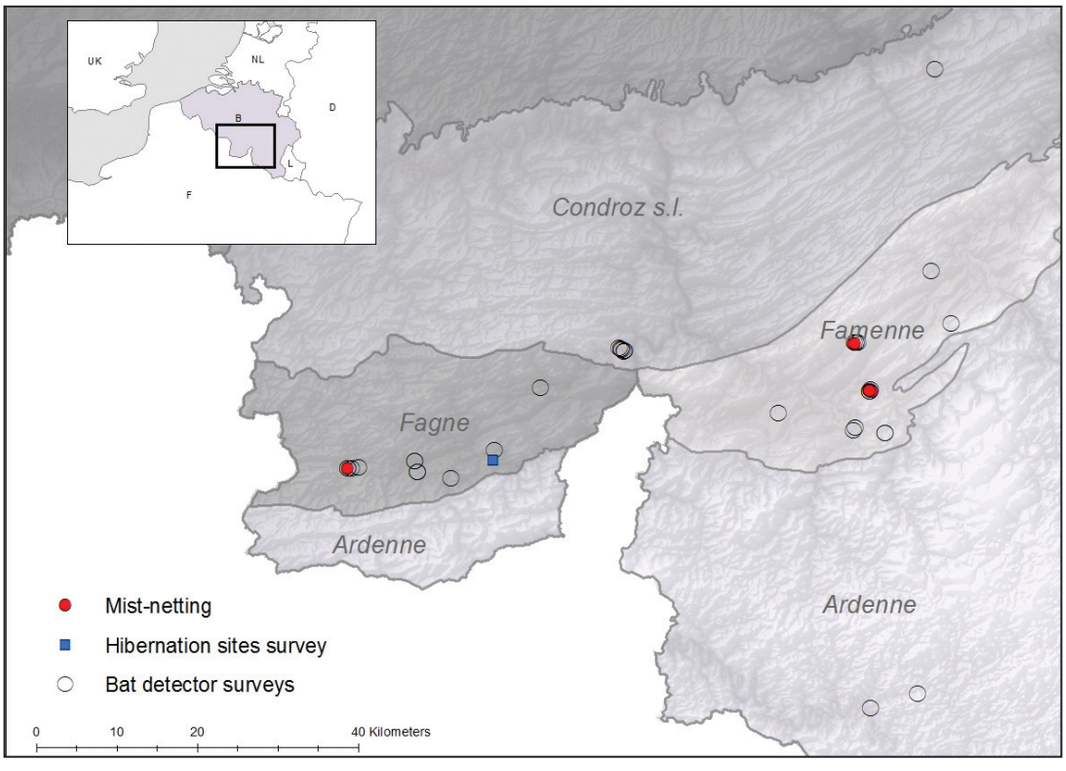

Fig. 1. – Map showing the locations of the Belgian records of *Myotis alcathoe*. Red circles: mist-net captures; open circles: bat detector records; blue squares: hibernation records.

Between 2011 and 2014, 13 *M. alcathoe* were captured during mist netting surveys in Wallonia (Table 1; Fig. 1). These individuals were all identified based on morphological characteristics (6). The capture of a post-lactating female and several juvenile bats (based on epiphyseal plates and secondary sexual characteristics) shows that a reproducing population is present in this region. During summer, the species has been captured in two old growth deciduous woodlands in Rochefort (forêt de Saint-Rémy; Province of Namur) (Fig. 2a) and Chimay (étang des prés de Virelles; Province of Hainaut). During the autumn swarming season the species has been captured at the entrance of a large natural cave in Rochefort from 2011 to 2014 (Grotte touristique de Rochefort; Province of Namur). This natural cave is a very important site for bats in this region, both for hibernation and for swarming. Eleven species have been captured there during the swarming season, including *M. mystacinus* and *M. brandtii*.

To confirm the morphological identification genetically, we collected faeces of three caught bats at this site in 2014 (Table 1). Bat faeces can be used for non-invasive genotyping to identify species (e.g. 15, 16). Each dropping was individually placed in a tube with silica gel to absorb humidity and hence preserve DNA (17) or in pure ethanol. Droppings that were stored in pure ethanol were air dried first for half an hour on a tissue paper prior to DNA extraction. DNA was extracted using the QIAamp Fast DNA Stool Mini kit (Qiagen), following the manufacturer's protocol except for some steps that were modified as described below (17) (step numbers correspond to the QIAamp Fast DNA Stool Mini Handbook, pp14-16, ver. 03/2014). A single dropping was placed individually in a 2 ml microtube as Step 1. After addition of the InhibitEX Buffer the dropping was squashed using a disposable pestle (Eppendorf) during Step 2 until completely homogenized. Incubation at 70 °C in Step 7 lasted 15 minutes in a Thermomixer at 750 rpm (Eppendorf). For Steps 9 and 11 centrifugation was performed at 7200 rpm. Step 13 was omitted. DNA was finally eluted in 100 µl Buffer ATE during Step 14 and this step was repeated by pipetting the eluate back on the column membrane to increase DNA yield.

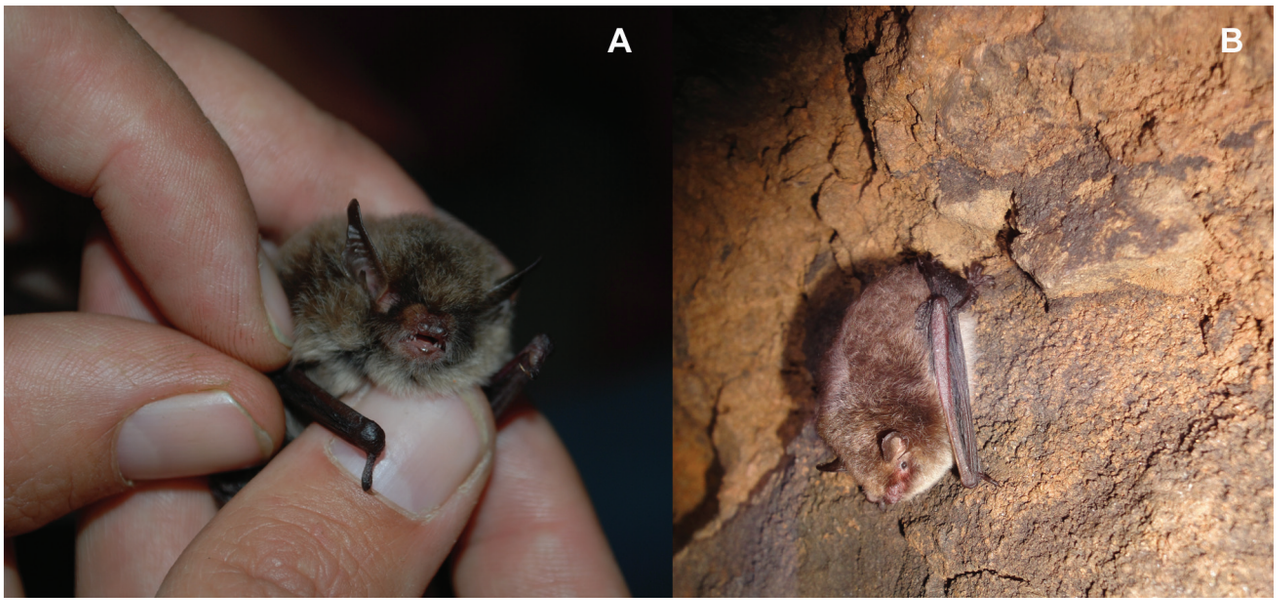

Fig. 2. – A. Mist-netted *M. alcathoe* in Forêt de Saint-Rémy (photo Pierrette Nyssen) **B**. *M. alcathoe* roosting in an underground tunnel (photo Bob Vandendriessche).

We amplified a 220 bp fragment containing 153 bp of the mitochondrial Cytochrome b gene using forward primer Bat_trnE31F (5'-TGACACGAAAAATCAYCGTTGT-3') and reverse primer Bat_cytb176R (5'-GTRTCTGATGTRTAGTGTATRGC-3'). PCRs were performed in 52 µL of reaction mixture containing 12 µL of extracted DNA, 0.4 µM of each primer, 1x Taq buffer with KCl, 2 mM $MgCl_2$, 200 µM of each dNTPs and 1.6 U Taq polymerase (Thermo Fisher Scientific). Each PCR was composed of an initial denaturation at 94 °C for 3 min; followed by 35 amplification cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s), followed by a final elongation at 72 °C for 10 min. Amplified DNA was purified using the ExoSAP-IT method (Affymetrix Inc.). Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) in a 10 µL volume containing 4.4 ng of purified DNA, 0.4 µM of forward or reverse primer, 0.5x Ready Reaction mix and 0.5x Sequencing buffer. Sequencing of both strands was performed with a cycling profile of 35 cycles of 10 s denaturation at 96 °C, 5 s annealing at 50 °C and 4 min elongation at 60 °C. After purification with the BigDye XTerminator Purification kit (Life Technologies) products were analyzed on an ABI 3500 genetic analyzer (Life Technologies). Sequences for the three bats were identical (EMBL Accesion No. LN864496) and an NCBI BLAST search showed that the 153 bp Cytb part was only identical to a set of *M. alcathoe* haplotypes (EU541661, EU541662, EU541663; 7).

Secondly, we sequenced 1365 bp of a 1460 bp fragment containing the complete ND1 gene (3) for one of these individuals (table 1, EMBL Accesion No. LN864497) following Jan et al. (2010) (13). Across the sequenced region, the haplotype was identical to the ND1 haplotype obtained from the Hungarian samples of *M. alcathoe* (AY027835 and AY027836; 3). This haplotype has been recorded in the Iberian peninsula (18) and across Western and central Europe (7, 9, 12, 13, 19), while a number of different closely related haplotypes have been observed in the Balkans and Asia minor (3, 20).

Up to now, only one roost site of *M. alcathoe* has been recorded. At the beginning of April (8/4/2012) a torpid individual was observed in an old tunnel in Viroinval (Province of Namur; Fig. 2b). This site – situated in an old growth riparian woodland – is likely used as a hibernation site or a transit roost by the species. Annually, up to 10 bat species are counted here during hibernation, among them ca. 5-10 individuals of 'whiskered bat' (*M. mystacinus/brandtii/alcathoe*). During a preliminary survey in the swarming season both *M. mystacinus* and *M. brandtii* were already captured at this tunnel (Dekeukeleire D, unpublished data).

Furthermore, several bat detector recordings in southern Belgium can be attributed to *M. alcathoe*. The first observation was made on the 29th of May 2008, in the wooded river valley between Hermeton-sur-Meuse and Soulme (Province of Namur). Using a night vision camera (Night Mariner 150, ITT, New York, US), a small *Myotis* bat could be observed foraging a few meters above the river Hermeton under overhanging branches. Ultrasound recordings were made with a D1000x bat detector (Pettersson Elektronik AB, Uppsala, Sweden) and analysed in the BatSound Pro 3.3 software package (Pettersson Elektronik AB, Uppsala, Sweden). Signals (n: 20) had the following characteristics (mean ± SD): duration 2.87 ± 0.45 ms, pulse interval 65.85 ± 15.95 ms, start frequency 118.65 ± 2.89 kHz, end frequency 42.55 ± 2.80 kHz, peak frequency 59.70 ± 8.11 kHz, sigmoid ('S'-) shape with upper and lower inflexion points at 59.70 ± 2.28 and 50.60 ± 2.24 kHz respectively (Fig. 3). These characteristics correspond well with the description by von Helversen et al. (2001) (3) and Barataud (2012) (21). The only other European species using echolocation calls that consistently end above 40 kHz is the Geoffroy's bat (*M. emarginatus*) (21, 22), but this species generally uses very high starting frequencies, up to 160 kHz (23). Moreover, *M. emarginatus* has linear-shaped echolocation

calls (duration < 3 ms) in confined spaces. The presence of sigmoid shapes and the absence of very high start frequencies, even though the bat flew within close proximity of the detector, points to *M. alcathoe* (3, 21). Additional detector surveys in 2011 - 2013 confirmed flight activity of *M. alcathoe* in the valley of Hermeton from April to October. Similar bat detector recordings were made in Bertrix (Province of Luxembourg), Villers-le-Temple (Province of Liège) and the wider surroundings of the capture sites in Rochefort (Province of Namur) and Virelles (Province of Hainaut) (Fig. 1).

These findings led us to re-identify all Belgian specimens of *M. mystacinus* (n: 124) and *M. brandtii* (n: 53) in the collection of the Museum of Natural Sciences (IRSNB) based on skull characteristics (3, 19, 24). However, no additional *M. alcathoe* specimens could be discovered.

Our records indicate that *M. alcathoe* is a resident species in the southern part of Belgium. Its presence has most likely gone undetected due to its similarity to other small *Myotis* species and its relatively recent description.

Recently, BOGDANOWICZ et al. (2012) (8) indicated possible high levels of hybridization between *M. mystacinus, M. brandtii* and *M. alcathoe* at swarming sites in Poland. Nuclear microsatellite markers indicated that 6.5 to 30.4 % of the *M. alcathoe* identified based on mtDNA were possible hybrids. Morphologically, the majority of these hybrids followed their mtDNA identification, although some showed intermediate phenotypes. As in other studies (e.g. 7, 18, 23) we have only used mitochondrial markers and morphological characteristics, and thus we cannot rule out the presence of hybrids. However, *M. alcathoe* is widely distributed in Europe and occurs in neighboring regions (6), and moreover, our observations show reproduction in Belgium. The probability that *M. alcathoe* does not occur in Belgium, and that our records only represent hybrids, thus seems very small.

The habitat where *M. alcathoe* has been observed in Belgium – natural old growth deciduous forests and caves - is very similar to their habitat in other European regions (eg. 7, 8, 13). Up to now, most of the records are from the southern part of the Fagne-Famenne region in Wallonia (Fig. 1). This region – characterized by the presence of Devonian limestone – is a biodiversity hotspot in Belgium, and several plant and arthropod species from Mediterranean and continental biogeographic regions occur here (e.g. 25, 26). This occurrence pattern is quite similar to the distribution in Saxony-Anhalt (Germany), where the most northeastern German records of *M. alcathoe* have been noted (7). However, there are also bat detector records in the Condroz and in a wooded river valley in

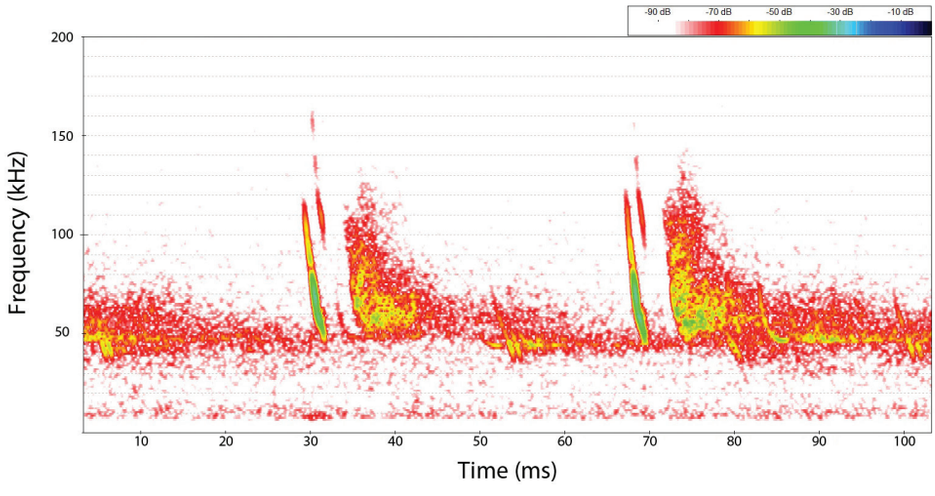

Fig. 3. – Echolocation signals of *M. alcathoe* recorded in Hermeton-sur-Meuse on 29/05/2008 (recording nr M00037 Marc Van de Sijpe).

the Ardennes. Additional surveys in old growth forests and mist netting at swarming sites could reveal additional observations and clarify the range of this species in Belgium.

Bats are considered to be highly threatened due to habitat loss, pesticide use and anthropogenic disturbances (6). In southern Belgium, hibernation census data indicate a strong decrease in both species diversity and bat abundance at underground sites over the past 50 years (27). At this point, it is too early to determine the conservation status of *M. alcathoe* in Belgium, but it appears to be rare. As a forest specialist with a limited distribution, *M. alcathoe* could be regarded as a priority species for conservation plans.

ACKNOWLEDGEMENTS

All bats were captured and handled under license from the Belgian authorities (SPW-DNF-DGO3 permit réf déro 2013/RS/n°15 and dero 2014/RS/n°12). We would like to thank the many volunteers that helped with the fieldwork, especially those from Plecotus – Natagora, Jeunes & Nature and Vleermuizenwerkgroep – Natuurpunt. The coordination of bat field monitoring is funded by the public service of Wallonia (DGO3 - Contract "Convention pour l'aide au suivi des populations de chiroptères, la mise en place d'actions en faveur des chauves-souris et la sensibilisation et l'information du public"). Bat monitoring in Wallonia is conducted in close collaboration between Plecotus – Natagora and the public service of Wallonia.

We are very grateful to the Genetic Diversity lab technicians (INBO), Bart Hellemans and Filip Volckaert (KU Leuven) for assistance with the genetic analyses. We would also like to thank Christian Dietz for confirming the identification of the hibernating bat, and Alex Lefevre and Michel Barataud for their help with acoustic bat identification. Furthermore, we would like to thank Thierry Kervyn and Sébastien Puechmaille and an anonymous reviewer for useful comments on the first draft of the manuscript.

REFERENCES

1. Mayer F. & von Helversen O (2001). Cryptic diversity in European bats. Proceedings of the Royal Society B 268, 1825–1832.
2. Mayer F, Dietz C & Kiefer A (2007). Molecular species identification boosts bat diversity. Frontiers in Zoology, 4: 4.
3. von Helversen O, Heller K-G, Mayer F, Nemeth A, Volleth M & Gombkötö P (2001). Cryptic mammalian species: a new species of whiskered bat (*Myotis alcathoe*, n. sp.) in Europe. Naturwissenschaffen, 88: 217-223.
4. Ruedi M, Stadelmann B, Gager Y, Douzery EJP, Francis CM, Lin LK, Guillén-Servent A & Cibois A (2013). Molecular phylogenetic reconstructions identify East Asia as the cradle for the evolution of the cosmopolitan genus *Myotis* (Mammalia, Chiroptera). Molecular Phylogenetics and Evolution, 69: 437–449.
5. Boston ESM, Hanrahan N, Puechmaille SJ, Buckley DJ, Lundy MG, Scott DD, Prodohl PA, Montgomery WI & Teeling EC (2011). A rapid PCR-based assay for identification of cryptic *Myotis* sp. (*M. mystacinus, M. brandtii and M. alcathoe*). Conservation Genetics Resources, 3: 557-563.
6. Dietz C, von Helversen O & Nill D (2007). Handbuch der Fledermäuse Europas und Nordwestafrikas. Biologie, Kennzeichen, Gefährdung. Franckh-Kosmos Verlags GmbH, Stuttgart.
7. Niermann N, Biedermann M, Bogdanowicz W, Brinkmann R, Le Bris Y, Ciechanowski M, Dietz C, Dietz I, Estók P, von Helversen O, Le Houédec A., Paksuz S, Petrov BP, Ozkan B, Piksa K, Rachwald A, Roué SY, Sachanowicz K, Schorcht W, Tereba A & Mayer F (2007). Biogeography of the recently described *Myotis alcathoe* von Helversen and Heller, 2001. Acta Chiropterologica, 9: 361-378.
8. Lučan RK, Andreas M, Benda P, Bartonička T, Březinová T, Hoffmannová A, Hulová S, Hulva P, Neckářová J, Reiter A, Svačina T, Šálek M & Horáček I (2009). Alcathoe Bat (*Myotis alcathoe*) in the Czech Republic: Distributional Status, Roosting and Feeding Ecology. Acta Chiropterologica, 11: 61-69.
9. Bogdanowicz W, Piksa K & Tereba A (2012). Hybridization hotspots at bat swarming sites. Plos One, 7: e53334.

10. Ohlendorf B (2009). Aktivitäten der Nymphenfledermaus (*Myotis alcathoe*) vor Felsquartieren und erster Winternachweis im Harz (Sachsen-Anhalt). Nyctalus, 14: 149-157.
11. Sachanowicz K, Mleczek T, Gottfried T, Ignaczak M, Piksa K & Piskorski M (2012). Winter records of *Myotis alcathoe* in southern Poland and comments on identification of the species during hibernation. Acta Zoologica Cracoviensia, 55(1): 97-101.
12. Ruedi M, Jourde P, Giosa P, Barataud M & Roué Y (2002). DNA reveals the existence of *Myotis alcathoe* in France (Chiroptera: Vespertilionidae). Revue Suisse de Zoologie, 109: 643-652
13. Jan CMI, Frith K, Glover AM, Butlin RK, Scott CD, Greenaway F, Ruedi M, Frantz AC, Dawson DA, Altringham JD (2010). *Myotis alcathoe* confirmed in the UK from mitochondrial and microsatellite DNA. Acta Chiropterologica, 12: 471-483.
14. Gessner B (2012). Teichfledermaus (*Myotis dasycneme* Boie, 1825) und Nymphenfledermaus (*Myotis alcathoe* Helversen & Heller, 2001), zwei neue Fledermausarten für Luxemburg. Bulletin de la Société des naturalistes Luxembourgeois, 113: 137-140.
15. Boston ESM, Puechmaille SJ, Scott DD, Buckley DJ, Lundy MG, Montgomery WI, Prodohl PA & Teeling EC (2012). Empirical assessment of non-invasive population genetics in bats: comparison of DNA quality from faecal and tissue samples. Acta Chiropterologica, 14: 45–52.
16. Puechmaille SJ & Teeling EC (2014). Non-invasive genetics can help find rare species: a case study with *Rhinolophus mehelyi* and *R. euryale* (Rhinolophidae: Chiroptera) in Western Europe. Mammalia, 78(2): 251-255.
17. Puechmaille SJ, Mathy G & Petit EJ (2007). Good DNA from bat droppings. Acta Chiropterologica, 9(1): 269-276.
18. Agirre-Mendi P, García-Mudarra J, Juste J & Ibanez C (2004). Presence of *Myotis alcathoe* Helversen and Heller, 2001 (Chiroptera: Vespertilionidae) in the Iberian Peninsula. Acta Chiropterologica, 6: 49-57.
19. Spitzenberger F, Pavlinic I & Podnar M (2008). On the occurrence of *Myotis alcathoe* von Helversen and Heller, 2001 in Austria. Hystrix Italian Journal of Mammalogy, 19: 3-12.
20. Çoraman E, Furman A, Karataş A, Bilgin R (2013). Phylogeographic analysis of Anatolian bats highlights the importance of the region for preserving the Chiropteran mitochondrial genetic diversity in the Western Palaearctic. Conservation Genetics, 14: 1205-1216.
21. Barataud M (2012). Ecologie acoustique des chiroptères d'Europe. Identification des espèces, étude de leurs habitats et comportements de chasse. Biotope Editions, Mèze – Muséum national d'Histoire naturelle, Paris.
22. Walters CL, Freeman R, Collen A, Dietz C, Fenton MB, Jones G, Obrist MK, Puechmaille SJ, Sattler T, Siemers BM, Parsons S & Jones KE (2012). A continental-scale tool for acoustic identification of European bats. Journal of Applied Ecology, 49: 1064-1074.
23. Schumm A, Krull D & Neuweiler G (1991). Echolocation in the notch-eared bat (*Myotis emarginatus*). Behavioral Ecology and Sociobiology, 28: 255-261.
24. Pavlinic I, Tvrtkovi N & Podnar M (2012). Preliminary data on genetics and morphometrics of *Myotis alcathoe* (Chiroptera, Vespertilionidae) in Croatia. Mammalia, 76: 331–334
25. Maes D, Gilbert M, Titeux N, Goffart P & Dennis RLH (2003). Prediction of butterfly diversity hotspots in Belgium: a comparison of statistically focused and land use-focused models. Journal of Biogeography, 30: 1907-1920.
26. Piqueray J, Bisteau E, Cristofoli S, Palm R, Poschlod P & Mahy G (2011). Plant species extinction debt in a temperate biodiversity hotspot: Community, species and functional traits approaches. Biological Conservation, 144: 1619-1629.
27. Kervyn T, Lamotte S, Nyssen P & Verschuren J (2009). Major decline of bat abundance and diversity during the last 50 years in southern Belgium. Belgian Journal of Zoology, 139: 124-132.

Received: January 30th, 2015
Accepted: July 15th, 2015
Branch editor: Peter Galbusera