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Selective use of forest habitat by Biłgoraj horses

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ABSTRACT. Primitive horses are quite often kept in nature reserves with access to the forest, which they sometimes penetrate to use the vegetation. Horses, as grazers, use specific foraging and anti-predator strategies that differ from typical browsers. The aim of the study was to assess the factors influencing the pattern of forest use by Bilgoraj horses. We hypothesized that the essential factors influencing their pattern of foraging are: browse abundance, distance to pasture, and openness of the habitat. Data were collected at the Biłgoraj Horse-Breeding Centre near Janów Lubelski, Poland. The horses browsed significantly more on woody vegetation in parts of the forest more exposed to sunlight and more abundant in browse material (especially containing preferred species). Distance to the main pasture had a significant effect upon browsing intensity only when an interaction with the abundance of preferred browse was considered.

KEY WORDS: Biłgoraj horse, browse, forest, habitat openness

INTRODUCTION

Grazing by horses is increasingly becoming the preferred mode of natural maintenance of meadows that are no longer under agricultural practices. It controls the growth of woody vegetation in grassland communities as it helps in the delay or even prevention of the processes of secondary succession (Borkowski, 2002). Due to their adaptability primitive races of horses are preferred for such tasks. These horses are kept in reserves with access to the forest, which they occasionally penetrate to forage on its vegetation (Jaworski, 2003; Stachurska et al., 2006). There have been no prior studies on patterns of habitat use in forests. However, there were a number of studies focusing on grazers in relation to grassy environments (e.g. Coughenour, 1991; Bailey et al., 1996; Edouard et al., 2009), woody species succession on meadows (Green & Kauffman, 1995; Carmel & Kadmon, 1999), and their impact on forest undergrowth, and the growth of tree seedlings (van Uytvanck & Hoffmann, 2009; Wassie et al., 2009; Törn at al., 2010; Bobiec et al., 2011). Some studies also considered the issue of bark stripping by grazers (Kuiters et al., 2006; Klich, 2009). Although woody vegetation constitutes a marginal percentage in the diet of horses (Cosyns et al., 2001), high density of animals, which is common in reserve conditions, may lead to a significant impact on tree stands (Jorritsma et al., 1999). Better knowledge regarding the factors influencing decisions by horses regarding their foraging patterns would be useful in the management of free ranging animals and could help to minimize damage to forest stands.

Both wild (King, 2002) and domestic horses (Jorritsma et al., 1999; Cosyns et al., 2001; Kuiters et al., 2006) use the forest habitat to a limited degree. Horses do not enter forests only to search for food (Cosyns et al., 2001; Kuiters et al., 2006). Other motivations play a role such as seeking protection against insects and high temperatures, as well as looking for substrates to rub against (Jezierski & Jaworski, 1995; King, 2002). The foraging pattern of horses in a forest results from both the specific structure of patches of woody vegetation and their specific foraging strategy. There is a lack of knowledge regarding the criteria the horses use when feeding upon woody vegetation. Further, it is not clear whether

abundance of browse and browse preference play a role. Following optimal foraging theory, one would expect horses – being bulk feeders – to graze upon taller vegetation to take in more food with minimized energy costs (Edouard et al., 2009). Horses require higher amounts of food than do ruminants, since they are hindgut fermenters, digesting food more rapidly but relatively less efficiently (Illius & Gordon, 1992). Quite opposite to this expectation, some evidence indicates that horses tend to graze on lower vegetation (Fleurance et al., 2001; Lamoot et al., 2005) and have a strategy of maintaining parts of the meadow in good quality by permanent grazing just like other grazers (Bakker et al., 1983; Fleurance et al., 2001). Although horses are able to utilize low quality herbage (Gudmundsson & Dyrmundsson, 1994; Chodkiewicz & Stypiński, 2011), their selection of food of better quality based on digestibility of energy aligns with optimal foraging theory. Given the diversity of species in grassland communities, even within an individual patch there will be spatial diversity of food quality resulting in selectivity of the grazers (Searle & Shipley, 2010). With regard to browse material, the quality may differ strongly between tree species, individual trees, and even within a tree (Suomella & Ayres, 1995; Touré et al., 1998). This difference in quality seems to affect foraging by browsers, but apparently grazers use woody vegetation much more homogenously (Searle & Shipley, 2010). As grazers, horses are less selective with regard to browse quality relative to browsers such as red deer (van Wieren, 1996). On the other hand, horses have a lower ability to detoxify plant secondary compounds than do browsers (McNab, 2002). There is even some evidence for browse selectivity in horses (Skiwski & Klich, 2012). Polish konik in the Eastern Carpathians tend to browse on woody vegetation characteristic of more open parts of the forest (Skiwski & Klich, 2012). Massive abundance of browse in the forest habitat affects the amount of light available to lower parts of the forest. In some cases, the presence of undergrowth also fundamentally changes the light conditions (Conway et al., 1997). Those changes may be connected with lower lateral visibility (due to the density of twigs) or just limit the amount of sunlight reaching the ground vegetation. Horses are typical open-environment species. This is an anti-predator strategy allowing them to scan their surroundings for any predator. Horses in captivity show a high plasticity to artificial conditions while retaining most of their natural reactions including the anti-predator behaviour (Hansen et al., 2001; Estevez et al., 2007). The possibility of an unexpected attack makes ungulates vulnerable to predators, resulting in avoidance of forest habitats by some species (Pépin et al., 1996), and a quicker flight reaction in highly risky habitats (LaGory, 1987; Taraborelli et al., 2012). Moreover, horses need a relatively long time to adapt to the dark when moving between extreme light conditions (Hanngi & Ingersoll, 2009). The distance to pasture is another factor relating to the use of forest habitat by horses. Horses spend more than half of the day grazing on pasture (Duncan, 1992; Fleurance et al., 2001). Their food choice is based upon quality of the grassy vegetation, a factor that also determines their level of woody vegetation use (Cosyns et al., 2001). Horses were found to engage in debarking of trees and shrubs of preferred species, and foraging was much more intense along the edge of a tree stand and at solitary trees or shrubs in meadows (Klich, 2009). There have been similar findings for other ungulate species underlining the influence of spatial distribution of vegetation upon foraging behaviour within substitute habitats (Clarke et al., 1995; Hester & Baillie, 1998). From optimal foraging theory, a meadow as a main feeding site, may optimally fulfill the requirements of horses regarding food and safety. When foraging in woodlands, horses expend energy for translocation and spend more time in a less open and thus more risky environment. On the other hand, there is some evidence that for typical forest ungulates, which perceive open areas as risky, the distance to the forest edge is a factor that determines the level of meadow use (e.g. Aulak & Babińska-Werka, 1990). Foraging and anti-predator strategies may represent antagonistic needs and

motivations in individuals, in both the wild and captivity. Although there are other mechanisms mitigating this antagonism (e.g. CLARK & DUKAS, 1994), the predation risk usually reduces the foraging efficiency (BROWN, 1999; HOWERY & DELIBERTO, 2004; GUILLEMAIN et al., 2007). We expect that in forests, which for horses are a non-standard habitat, horses adapt their foraging strategy and anti-predator strategy. This study set out to assess the factors influencing forest use pattern of Biłgoraj horses, a race very closely related to Polish koniks. We hypothesize that three factors have an influence on forest habitat use by horses: (a) the abundance of browse; (b) the distance to the pasture; and (c) the light conditions that represent the openness of habitat. Locations that are safe (opened and close to pasture), and have an abundance of attractive food sites, should be then used by horses more frequently and for a longer time. By contrast, any inconvenience related to the habitat structure or any factor causing a decrease of food quality should shorten the time spent there by horses for grazing. We tested three factors that could influence the horses' choice in selecting particular plots for feeding:

1. The abundance of browse – represented by two variables: (a) Preferred browse – total number of twigs of preferred woody species (in each plot); we expected this variable to reflect the strength of tendency in choice of patch selection. (b) Other browse –total number of other twigs available within each plot (randomly taken and not preferred),
2. Distance to the pasture – represented by each plot distance to forest edge,
3. Openness of the habitat – represented by Trans Total value in each plot.

MATERIALS AND METHODS

Data was collected in Szklarnia Village, Biłgoraj Horse-Breeding Centre near Janów Lubelski in the East of Poland between September 5 and 10, 2011. Horses in the breeding center were kept in various conditions. Stallions were stabled with daily access to pastures, while mares and yearlings were free ranging in enclosures. Depending on the season, the group of mares with yearlings had access to meadows during spring, meadows with hay provided ad libitum during winter, and meadows with access to forest during summer and autumn. In the summer-autumn enclosure, the meadow was bordered directly by a forest patch about 100 m wide and 600 m long. Two habitats were distinguished within this forest: fresh mixed coniferous forest and alder swamp forest. Both habitats were spatially mixed and horses were interchangeably using them. Two months after the release of 20 horses into a summer-autumn enclosure, eight parallel transects in 50 m intervals were delimited within the forest habitat. Maximal transect length was 100 m but depended on the accessibility of the area for horses. For instance, transects were shorter if they reached the fence of the enclosure. Consequently, transect length varied from 30 m to 100 m. Along the transects, 5 m x 2 m plots were defined at 10 m intervals, starting from the edge of the forest. The total number of plots equalled 72. However, three plots were excluded from further analyses because of low accessibility for horses. This was due to felled trees or high level of water. Within each plot that was browsed by horses, the remaining twigs of trees and shrubs that were up to 2 m above the ground were counted, and categorized by tree or shrub species.

Based on the number of browsed and not consumed twigs, the Jacobs' selectivity index was calculated for all tree and shrub species to quantify the preference of horses for each species (JACOBS, 1974). The statistical significance of the Jacobs' selectivity index was verified by chi square test, comparing the number of twigs browsed and not consumed for each plant species separately (df = 1). To quantify the openness of the habitat, a photo was taken in the center of each plot with a fish-eye lens, directed vertically upwards at 1 m above the ground. Pictures were elaborated with Gap Light Analyzer. We used Trans Total value, which represented the amount of direct and diffused solar radiation passing through the canopy and topographic mask (FRAZER et al., 1999). The Trans Total value

represented the visual openness of the forest site at the height of one meter.

Statistical analyses using Statistica 9.0 software were performed to identify the factors that could influence the selective use of forest by horses. The dependent variable representing the browsing behaviour of the horses was the number of browsed twigs within each plot. Plots with less than 20 twigs of preferred browse or other browse were excluded from the analyses as it was assumed that low amounts of available browse would not be sufficiently attractive for horses. Out of 72 delimited plots along the transects, data from 49 plots were compared (after excluding plots with less than 20 twigs of preferred browse or other browse, and low accessibility for horses). In order to assess the influence of each factor upon foraging pattern of horses in the forest and to take mutual interactions and nonlinear relationships into consideration, we used a Response Surface Regression model in GRM (General Regression Models).

RESULTS

A total number of 29,777 browsed and not browsed twigs from 22 tree and shrub species were registered within all plots (Tab. 1). The highest number of twigs belonged to *Frangula alnus*, *Salix sp.*, *Abies alba* and *Alnus glutinosa*. Horses mainly browsed on *Frangula alnus* and *Abies alba*. According to the Jacobs' selectivity index, horses preferred six tree species, mainly deciduous and one coniferous – *Abies alba*. Among shrubs, horses preferred *Frangula alnus*, *Rubus sp.* and *Crataegus sp.* Other analyzed species were avoided by horses. In the case of *Padus avium*, the negative value was close to zero, and an intake of its browse should be regarded as random. Two other avoided species, *Populus tremula* and *Rosa canina*, were present in very low amounts and consequently the Jacobs' index did not show their real preference status.

When both habitats in this study – i.e. fresh mixed coniferous forest and alder swamp forest – were treated separately, we found that the preference towards browse in both habitats was limited by site openness or exposure (Fig. 1). The horses preferred *Tilia cordata* in the fresh coniferous forest and avoided it in the alder swamp forest, where that species was only available in one plot with limited openness. In the case of many other tree or shrub species, the direction of the preference of horses for them changed after excluding plots with low degree of openness.

Among all 49 analyzed plots, horses browsed unevenly. Two of the three tested factors had a significant independent influence on foraging pattern of horses (Tab. 2). There also occurred significant interactions among variables; in all cases preferred browse abundance (PB) interacted with all other variables. E.g. distance to the pasture (DP) interacted with PB, and had then a significant influence on foraging pattern of horses. Although, there are no statistically significant correlations among variables, a specific trend between them is apparent (Fig. 2). The amount of preferred browse tends to increase together with increasing distance to the pasture. This interaction reflects a mutual relationship between PB and DP, so the sole significant influence of DP on foraging pattern of horses was not found.

The interaction between habitat openness (OH) and the amount of preferred browse (PB) increased the joint role of both variables in the horses' feeding choices. However, no relationship between the variables was found. The third example of such interaction is one between preferred browse (PB) and other browse (OB). This interaction is a result of priorities in the feeding behaviour of horses, as they first search for preferred food and then tend to use mostly the available composition of browse.

Total abundance of browse as well as preferred browse and other browse determined most of the spatial decisions, but the fraction of available preferred browse was most important. This contribution was also shown in the significant

TABLE 1

Browse intake and its preference by Biłgoraj horses (Jacobs' index) and statistical significance (Chi-square test with Yates' correction), * as a percentage of all browsed twigs or all twigs available.

	Number of browsed twigs		Number of available twigs		Jacobs' Index	Chi square	p
	N	[%]*	N	[%]*			
Species							
Trees							
Abies alba	765	0.216	2922	0.098	0.433	444	0
Acer platanoides	2	0.001	36	0.001	-0.364	0.66	0.416
Alnus glutinosa	84	0.024	3309	0.111	-0.675	264.05	0
Betula pendula	137	0.039	746	0.025	0.22	22.11	0
Betula pubescens	61	0.017	236	0.008	0.373	29.81	0
Carpinus betulus	25	0.007	478	0.016	-0.394	16.69	0
Malus sylvestris	41	0.012	199	0.007	0.27	9.87	0.002
Fagus sylvatica	107	0.03	1700	0.057	-0.321	44.28	0
Padus avium	153	0.043	1419	0.048	-0.052	1.34	0.247
Padus serotina	4	0.001	69	0.002	-0.346	1.54	0.214
Picea abies	39	0.011	723	0.024	-0.382	24.45	0
Pinus sylvestris	33	0.009	410	0.014	-0.196	4.48	0.034
Populus tremula	0	0	4	0	-1	0.01	0.904
Quercus robur	173	0.049	489	0.016	0.509	168.71	0
Salix sp.	223	0.063	5285	0.177	-0.526	300.96	0
Sorbus aucuparia	128	0.036	578	0.019	0.308	41.7	0
Tilia cordata	7	0.002	37	0.001	0.228	0.79	0.374
Shrubs							
Corylus avellana	33	0.009	1133	0.038	-0.616	76.72	0
Crataegus sp.	57	0.016	305	0.01	0.224	9.48	0.002
Euonymus europaeus	0	0	107	0.004	-1	11.69	0.001
Frangula alnus	1124	0.317	7988	0.268	0.117	37.48	0
Rosa canina	0	0	3	0	-1	0.11	0.735
Rubus sp.	351	0.099	1601	0.054	0.318	116.56	0

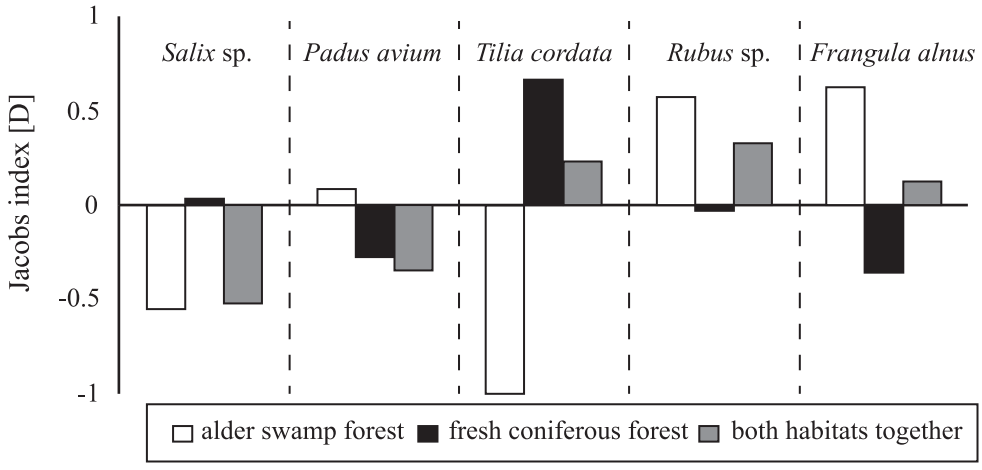

Fig. 1. – Jacobs' index in both studied forest habitats.

TABLE 2

Statistical summary of Response Surface Regression model using: distance to the pasture (DP), openness of the habitat (OH), preferred browse (PB), other browse (OB) to predict the frequency of browsing by horses (number of browsed twigs within each plot); $R^2 = 0.94$, R^2 (adjusted) = 0. 915, p = 0.000 for whole model.

variables	Beta (β)	Standard error (β)	t	R^2	p
DP	-0.084	0.051	- 1.62	0.333	0.113
DP^2	-0.036	0.066	- 0.58	0.597	0.565
OH	0.35	0.067	5.21	0.609	0.000
OH^2	0,021	0.051	0.62	0.346	0.539
PB	0.537	0.085	6.29	0.758	0.000
PB^2	0.137	0.078	4.2	0.714	0.000
OB	0.427	0.097	4.41	0.812	0.000
OB^2	-0.051	0.15	-1.15	0.922	0.259
DP*OH	-0.044	0.058	- 0.81	0.483	0.421
DP*PB	-0.189	0.06	- 2.52	0.516	0.016
OH*PB	0.399	0.079	4.17	0.721	0.000
DP*OB	0.014	0.089	0.27	0.776	0.787
OH*OB	-0.005	0.054	-0.06	0.399	0.949
PB*OB	0,384	0.06	3.68	0.513	0.001

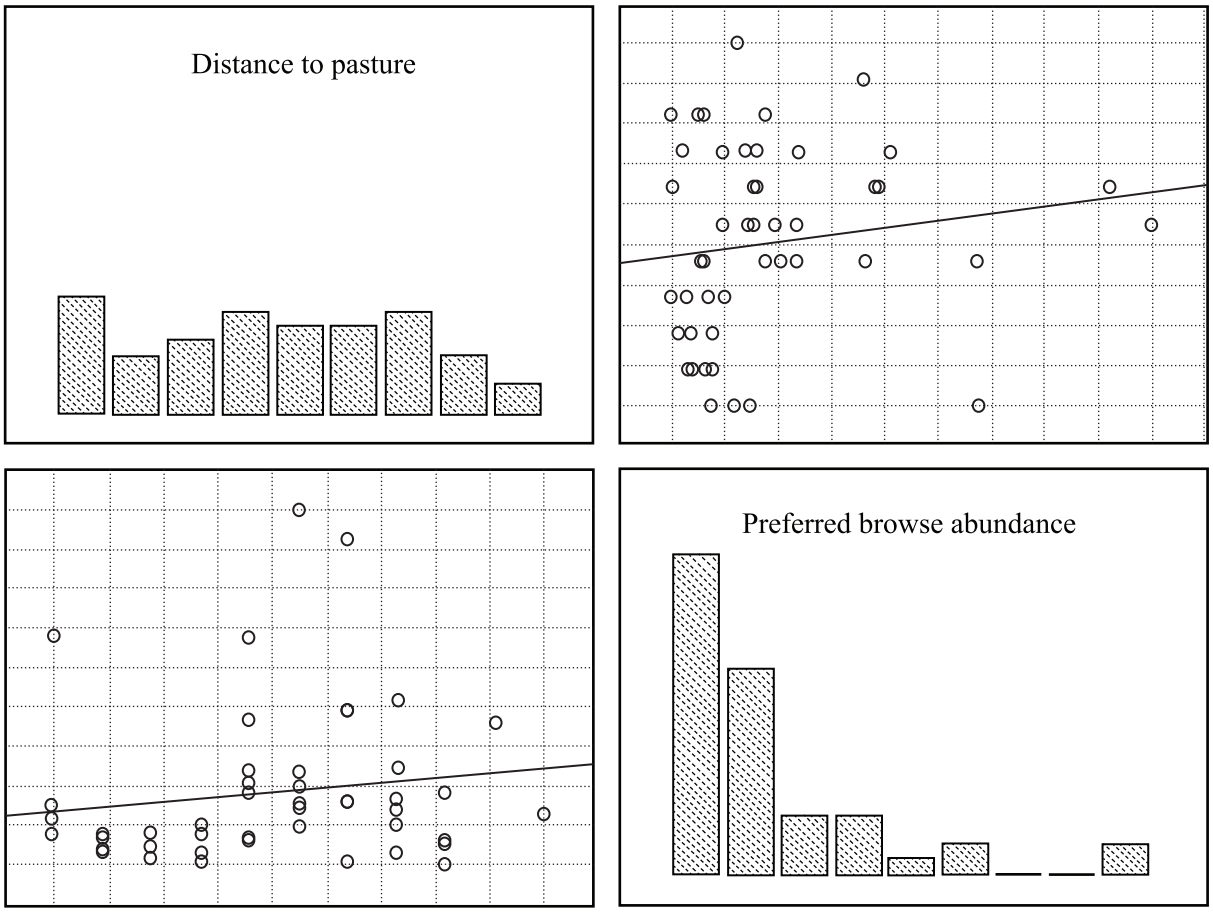

Fig. 2. – The scatter plot matrix of independent variables: distance to pasture and preferred browse abundance.

interactions of preferred browse with habitat openness and with presence of other woody species. This suggests that horses were actively looking for preferred woody species in open habitat and that they foraged more intensively in sites with a higher abundance of preferred species. Although the distance to the main pasture was not significant as a factor determining the spatial use of the forest habitat, this variable in combination with preferred browse, did show a negative influence on foraging intensity. The correlation between both parameters was low but their interaction caused the variable "distance to the pasture" to become significant in the model due to the high correlation between the parameter "preferred browse" and the dependent variable "number of browsed twigs". Although sole DP influence on foraging pattern was not statistically important, such interaction among variables caused an obvious tendency of horses to search for preferred twigs closer to pasture.

DISCUSSION

Although the Jacobs' index revealed a distinct preference of horses for almost half of the available tree and shrub species, the results showed that horses exhibit a dietary spectrum and food preferences dependent on local conditions. Konik horses, which are closely related to Bilgoraj horses, always showed a preference for *Rosa canina* and *Prunus spinosa*, and *Fagus sylvatica* and *Salix sp.* in conditions of high density of horses in the Bieszczady Mountains (Skiwski & Klich, 2012). *Betula sp.*, which was preferred in our study, was highly avoided there. The differences are probably not due to differences between races of horses but to environmental factors. Konik horses showed a high plasticity in woody and meadow vegetation (van Wieren, 1996; Chodkiewicz & Stypiński, 2011; Skiwski & Klich, 2012). In other analyses, koniks were actively looking for *Betula sp.*, and they also avoided *Salix sp.* and *Alnus sp.*, as in our study (Borkowski, 1997). The data indicate a wide dietary spectrum in Polish konik as well as Biłgoraj horses. Although both races show high preferences towards some woody species, they can also browse on many other species of second order of preference.

The results indicate that browse consumption of Biłgoraj horses is considerably dependent on site openness since they significantly preferred exposed sites. Where shrubs were abundant in the nature reserves in Belgium, Koniks used mainly shrubs, and their use of the forest habitat was only marginal (Hoffmann, 2002). Leaves and twigs exposed to sunlight contain more proteins, but also higher amount of tannins and other secondary plant metabolites (Hartley et al., 1997; Karolewski et al., 2011). These limit the nutritive value of plant tissues (Duncan & Poppi, 2010), but higher biomass and regeneration may induce ungulates to browse in forest gaps exposed to the light (Kuijper et al., 2009). According to these findings horses seem to follow a similar behaviour, particularly since they can feed on lower quality food (Gudmundsson & Dyrmundsson, 1994; Chodkiewicz & Stypiński, 2011) relative to donkeys (Cosyns et al., 2001) and compensate for lower energy intake by consuming higher quantities of food (Illius & Gordon, 1992; Clauss et al., 2010). Openness of habitat increases its importance for foraging behavior because of interaction with PB, which suggests an active search for preferred twigs by horses in more open parts of forest habitat.

The analysis of results has shown that, apart from habitat openness, horses are guided by the abundance of preferred woody species. Looking for patches of preferred browse, they choose a feeding station and forage there for a longer time. At these feeding stations they will indulge in bulk feeding with low selectivity, specific for grazer type feeding. This results in consumption of a high amount of twigs from other woody species normally randomly taken or even avoided. In consequence, horses attracted to a patch of preferred woody species also consume twigs of other species once there. Probably, the presence of PB in particular parts of the forest plays the initial role in choice, then the total abundance of

food (usually connected with habitat openness) is the factor that decides whether the animal will remain within the selected site. In this regard, the interaction among variables that may strengthen or diminish the attractiveness of a given habitat patch is important.

This foraging strategy overlaps with the antipredator strategy. Open sites in the forest offer better visibility, allowing for more time for retreat from or defense against eventual predators. For large herbivores living in grasslands, defense in such places would only imply flight (e.g. FITZGIBBON & LAZARUS, 1995). Although horses notice small stimuli better on overcast days than on sunny days (SASLOW, 1999), and maintain good visibility in the dim light, they require a relatively long time to adapt to the dark when moving from bright light conditions (HANGGI & INGERSOLL, 2009). This explains their tendency to avoid dark parts of the forest. Individuals that have lower energy demands due to reduced energy loss and a well-balanced food base, which is usually the case in captivity, have no reason to take risks and will probably tend to shift their foraging from risky to safe microhabitats (BROWN, 1999). The choice of more safe habitats results in foraging in open sites of the forest, higher amounts of food intake, occasionally also including not-preferred woody species. Further, monocotyledonous vegetation, which constitutes the main food base for horses, occurs mostly in parts of the forest exposed to sunlight (e.g. VOSPERNIK & REIMOSER, 2008). Thus, the use of open forest makes possible the use of abundant and fast recovering browse while allowing access of sunlight to the ground vegetation thereby promoting the growth of graminoids.

According to the results we obtained, browsing intensity depended on distance to the main feeding ground (pasture), but this relationship was only significant when considered as an interaction with PB. This can possibly be explained by the spatial distribution of the plots in different light conditions. Plots in the alder swamp forest were mostly much darker than those in fresh mixed coniferous forest, or than plots situated at the edge of the forest. Horses first had to cross the alder forest in order to reach the more open coniferous stand. The width of a strip of alder swamp forest varied along particular transects and this could have caused a trend towards higher abundance of PB with increasing distance to pasture. Moreover, transects often ended near the forest road where the degree of openness was very high and where the site was abundant with graminoids. Horses could spend a little more time in these places grazing and browsing, treating this area as a "supplementary pasture". This aspect could explain the weak significance of the factor "distance to the pasture" on the overall foraging pattern of horses, which was modified by specific local conditions. Therefore, we determined that horses were looking for preferred browse closer to the pasture, and the amount of PB was the main variable influencing their choice in the pattern of forest use.

CONCLUSIONS

We found a tendency of Biłgoraj horses to search for feeding sites abundant in browse (especially preferred species), more exposed to sunlight and close to the pasture. Using more open sites ensures their sense of security, gives access to fast-recovering browse, and stimulates the development of monocotyledonous species. They also follow a foraging strategy of maintaining good quality forage in selected patches. They forage on high amounts of food in open, quickly recovering sites. This suggests that in spite of some selectivity towards available food resources, their preference is more oriented towards patches in specific conditions (openness and browse abundance) than to singular woody species. Such selectivity has already been shown in grazers in grassy environments (SEARLE & SHIPLEY, 2010). We speculate that the more open are the available sites in the forest, the greater would be their penetration by horses and the longer would be the time spent there. Such pattern of habitat use would lead to uneven forest growth, and may stimulate changes in

undergrowth and ground flora towards shrub reduction and massive occurrence of grasses. Such process indeed occurred in the areas of Roztoczański National Park where Polish koniks roam, specifically in the more exposed tree stands (Wlizło & Szwed, 2007).

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REFERENCES

Aulak W & Babińska-Werka J (1990). Use of agricultural habitat by roe deer inhabiting a small forest area. Acta Theriologica, 35(1-2):121-127.

Bailey DW, Gross JE, Laca EA, Rittenhouse LR, Coughenour MB, Swift DM & Sims PL (1996). Mechanisms that result in large herbivore grazing distribution patterns. Journal of Range Management, 49(5):386-400.

Bakker JP, de Leeuw J & van Wieren SE (1983). Micro-patterns in grassland vegetation created and sustained by sheep-grazing. Vegetatio, 55(3): 153-161.

Bobiec A, Kuijper DPJ, Niklasson M, Romankiewicz A & Solecka K (2011). Oak (*Quercus robur* L.) regeneration in early successional woodlands grazed by wild ungulates in the absence of livestock. Forest Ecology and Management, 262(5):780-790.

Bokdam J, van Braeckel A, Werpachowski C & Znaniecka M (eds). (2002). Grazing as a conservation management tool in peatland. Report of a Workshop held 22-26 April 2002 in Goniadz (PL), Goniadz.

Borkowski M (1997). Koniki polskie w czynnej ochronie przyrody. In: Sokólska J (ed), Hodowla zachowawcza i użytkowa konika polskiego. Materiały sympozjum w Supraślu (13.06.1997) w ramach II spotkań z Naturą i Sztuką, Supraśl: 39-46.

Borkowski M (2002). Limiting bush encroachment at Biebrza marsh by Konik/Tarpan grazing. In: Bokdam J, van Braeckel A, Werpachowski C & Znaniecka M (eds), Grazing as a conservation management tool in peatland. Report of a Workshop held 22-26 April 2002 in Goniadz (PL), Goniadz:96-98.

Brown JS (1999). Vigilance, patch use and habitat selection: foraging under predation risk. Evolutionary Ecology Research, 1:49-71.

Carmel Y & Kadmon R (1999). Effects of grazing and topography on long-term vegetation changes in a Mediterranean ecosystem in Israel. Plant Ecology, 145(2):243-254.

Chodkiewicz A & Stypiński P (2011). Preferencje pokarmowe koników polskich wypasanych w Biebrzańskim Parku Narodowym. Woda-Środowisko-Obszary Wiejskie, 11(2):33-42.

Clarke JL, Welch D & Gordon IJ (1995). The influence of vegetation pattern on the grazing of heather moorland by red deer and sheep. I. The location of animals on grass/heather mosaic. Journal of Applied Ecology, 32(1):166-176.

Clauss M, Kaiser T & Hummel J (2010). The morphophysiological adaptations of browsing and grazing mammals. In: Gordon IJ *&* Prins HHT (eds), The Ecology of Browsing and Grazing. Springer-Verlag, Berlin, Heidelberg:47-88.

Conway DW, Parker AJ & Parker KC (1997). Understory light regime, shrub layer and sand pine (*P*In*us clausa*) regeneration in four scrub stands. American Midland Naturalist, 138(1):84-96.

Cosyns E, Degezelle T, Demeulenaere E & Hoffmann M (2001). Feeding ecology of Konik horses and donkeys in Belgian coastal dunes and its implications for nature management. Belgian Journal of Zoology, 131(Supplement 2):111-118.

Coughenour MB (1991). Spatial components of plant-herbivore interactions in pastoral, ranching, and native ungulate ecosystems. Journal of Range Management, 44(6):530-542.

Duncan AJ & Poppi DP (2010). Nutritional ecology of grazing and browsing ruminants. In: Gordon IJ *&* Prins HHT (eds), The Ecology of Browsing and Grazing, Springer-Verlag, Berlin, Heidelberg: 89-116.

Duncan P (1992). Horses and grasses. The nutritional ecology of equids and their impact on the Camargue. Springer-Verlag, New York.

Edouard N, Fleurance G, Dumont B, Baumont R & Duncan P (2009). Does sward height affect feeding patch choice and voluntary intake

in horses? Applied Animal Behaviour Science, 119(3-4):219-228.

Esteves I, Andersen I-L & Nævdal E (2007). Group size, density and social dynamics in farm animals. Applied Animal Behaviour Science, 103(3-4):185-204.

FitzGibbon CD & Lazarus J (1995). Antipredator behaviour of Serengeti ungulates: Individual differences and population consequences. In: Sinclair ARE & Arcese P (eds), Serengeti II: Dynamics, management and conservation of an ecosystem, University of Chicago Press, Chicago: 274-*296*.

Fleurance G, Duncan P & Mallevaud B (2001). Daily intake and the selection of feeding sites by horses in heterogeneous wet grasslands. Animation Resources, 50(2):149-156.

Frazer GW, Canham CD & Lertzman KP (1999). Gap Light Analyzer (GLA), Version 2.0: Imaging software to extract canopy structure and gap light transmission indices from true-colour fisheye photographs, users manual and program documentation. Copyright © 1999: Simon Fraser University, Burnaby, British Columbia, and the Institute of Ecosystem Studies, Millbrook, New York.

Green DM & Kauffman JB (1995). Succession and livestock grazing in a north-eastern Oregon riparian ecosystem. Journal of Range Management, 48(4):307-313.

Gudmundsson O & Dyrmundsson O (1994). Horse grazing under cold and wet conditions: a review. Livestock Production Science, 40:57-63.

Guillemain M, Arzel C, Legegneux P, Elmberg J, Fritz H, Lepley M, Pin C, Arnaud A & Massez G (2007). Predation risk constrains the plasticity of foraging behaviour in teals, Anas crecca: a flyway-level circumannual approach. Animal Behaviour, 73(5):845-854.

Hanggi EB & Ingersoll JF (2009). Stimulus discrimination by horses under scotopic conditions. Behavioural Processes, 82(1):45-50.

Hansen I, Christensen F, Hansen HS, Braastad B & Bakken M (2001). Variation in behavioural responses of ewes towards predator-related stimuli. Applied Animal Behaviour Science, 70(3):227-237.

Hartley SE, Iason GR, Duncan AJ & Hitchcock D (1997). Feeding behaviour of red deer (*Cervus elaphus*) offered Sitka spruce saplings (Picea sitchensis) grown under different light and nutrient regimes. Functional Ecology, 11:348-357.

Hester AJ & Baillie GJ (1998). Spatial and temporal patterns of heather use by sheep and red deer within natural heather/grass mosaics. Journal of Applied Ecology, 35(5):772-784.

Hoffmann M (2002). Experiences with grazing in Flemish nature reserves (N. Belgium). In: Bokdam J, van Braeckel A, Werpachowski C & Znaniecka M (eds), Grazing as a conservation management tool in peatland. Report of a Workshop held 22-26 April 2002 in Goniadz (PL), Goniadz: 49-53.

Howery LD & DeLiberto TJ (2004). Indirect effects of carnivores on livestock foraging behavior and production. Sheep & Goat Research Journal, 19:53-57.

Illius AW & Gordon IJ (1992). Modelling the nutritional ecology of ungulate herbivores: Evolution of body size and competitive interactions. Oecologia, 89(3):428-434.

Jacobs J (1974). Quantitative measurement of food selection. A modification of the forage ratio and Ivlev's Electivity Index. Oecologia, 14:413-417.

Jaworski Z (2003). Ocena warunków etologiczno-hodowlanych koników polskich utrzymywanych w systemie rezerwatowym. Wydawnictwo UWM, Olsztyn.

Jezierski T & Jaworski Z (1995). Reakcje obronne przed owadami u koników w warunkach pastwiska i rezerwatu leśnego. In: Jaczewski Z, Żurkowski M & Jaworski Z (eds), Biologia i hodowla zachowawcza konika polskiego. Materiały z ogólnopolskiej konferencji naukowej Popielno 18-19 maja 1995 r, Stacja Badawcza Rolnictwa Ekologicznego i Hodowli Zachowawczej PAN w Popielnie, Popielno:29-34.

Jorritsma ITM, van Hees AFM & Mohren GMJ (1999). Forest development in relation to ungulate grazing: a modeling approach. Forest Ecology and Management, 120:23-34.

Karolewski P, Jagodziński A & Grzebyta J (2011). Influence of tree age, needle age and location in the crown on the phenolic compounds content in needles of young Scots pines, Sylwan, 155(12):797-807.

King SRB (2002). Home range and habitat use of free-ranging Przewalski Horses at Hustai National Park, Mongolia. Applied Animal Behaviour Science, 78(2-4):103-113.

Klich D (2009). Analiza uszkodzeń kory spowodowanych przez konika polskiego w warunkach zagrodowych w Bieszczadach. Roczniki Bieszczadzkie, 17:307-317.

Kuijper DPJ, Cromsigt JPGM, Churski M, Adam B, Jędrzejewska B & Jędrzejewski W (2009). Do ungulates preferentially feed in forest gaps in European temperate forest? Forest Ecology and Management, 258:1528-1535.

Kuiters AT, van der Sluijs LAM & Wytema GA (2006). Selective bark-stripping of beech, *Fagus silvatica*, by free-ranging horses. Forest Ecology and Management, 222:1-8.

LaGory KE (1987). The influence of habitat and group characteristics on the alarm and flight response of white-tailed deer. Animal Behaviour, 35(1):20-25.

Lamoot I, Meert C & Hoffmann M (2005). Habitat use of ponies and cattle foraging together in a coastal dune area. Biological Conservation, 122(4):523-536.

McNab BK (2002). The physiological ecology of vertebrates: a view from energetics. Cornell University Press, New York.

Pépin D, Lamerenx F, Chadelaud H & Recarte J-M (1996). Human-related disturbance risk and distance to cover affect use of montane pastures by Pyrenean chamois. Applied Animal Behaviour Science, 46(3-4):217-228.

Saslow CA (1999). Factors affecting stimulus visibility for horses. Applied Animal Behaviour Science, 61(4):273-284.

Searle KR & Shipley LA (2010). The Comparative Feeding Behaviour of Large Browsing and Grazing Herbivores. In: Gordon IJ & Prins HHT (eds), The Ecology of Browsing and Grazing. Springer-Verlag, Berlin, Heidelberg:117-148.

Skiwski M & Klich D (2012). Spring and summer browsing by Polish konik in enclosures and free ranging conditions in the Bieszczady Mts, 156(10):792-800.

Stachurska A, Pięta M, Jaworski Z, Ussing AP & Pluta M (2006). Factors that influence coat hair length in primitive horses *(Equus caballus)*. Journal of Food, Agriculture and Environment, 4(1):215-219.

Suomella J & Ayres MP (1994). Within-tree and among-tree variation in leaf characteristics of mountain birch and its implications for herbivory. Oikos, 70(2):2112-222.

Taraborelli P, Gregorio P, Moreno P, Novaro A & Carmanchahi P (2012). Cooperative vigilance: the guanaco's (Lama guanicoe) key antipredator mechanism. Behavioural Processes, 91(1):82-89.

Touré SF, Michalet-Doreau B, Traoré E, Friot D & Richard D (1998). Occurrence of digestive interactions in tree forage-based diets for sheep. Animal Feed Science and Technology, 74(1):63-78.

Törn A, Siikamäki P & Tolvanen A (2010). Can horse riding induce the introduction and establishment of alien plant species through endozoochory and gap creation? Plant Ecology, 208(2):235-244.

van Uytvanck J & Hoffmann M (2009). Impact of grazing management with large herbivores on forest ground flora and bramble understorey. Acta Oecologica, 35(4):523-532.

van Wieren SE (1996). Do large herbivores select a diet that maximizes short-term energy intake rate? Forest Ecology and Management, 88:149-156.

Vospernik S & Reimoser S (2008). Modelling changes in roe deer habitat in response to forest management. Forest Ecology and Management, 255:530-545.

Wassie A, Sterck FJ, Teketay D & Bongers F (2009). Effects of livestock exclusion on tree regeneration in church forests of Ethiopia. Forest Ecology and Management, 257(3):765-772.

Wlizło B & Szwed W (2007). Roślinność Ostoi Konika Polskiego w Roztoczańskim Parku Narodowym. Prace Katedry Przyrodniczych Podstaw Leśnictwa Akademii Rolniczej im. Augusta Cieszkowskiego w Poznaniu, 2:1-117.

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Hurdles in investigating UVB damage in the putative ancient asexual *Darwinula stevensoni* (Ostracoda, Crustacea)

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ABSTRACT. Ostracoda or mussel-shrimps are small, bivalved Crustacea. Because of their excellent fossil record and their broad variety of reproductive modes, ostracods are of great interest as a model group in ecological and evolutionary research. Here, we investigated damage and repair from one of the most important biological mutagens, namely UVB radiation, in the putative ancient asexual ostracod *Darwinula stevensoni* from Belgium. We applied three different methods: the Polymerase Inhibition (PI) assay, Enzyme-Linked Immuno Sorbent Assay (ELISA) and dot blot. All three techniques were unsuccessful in quantifying UVB damage in *D. stevensoni*. Previous experiments have revealed that the valves of *D. stevensoni* provide an average UVB protection of approximate 60%. Thus, UVB damage could be too little to make quantitative experiments work. Additionally, variation between individual ostracods due to season and age most likely contributed further to the failure of the three used experimental approaches to quantify damage. In a second experiment, we investigated the influence of temperature on survival of *D. stevensoni* during UVB exposure. The estimated relative lethal UVB dose at 4°C was with 50 kJ/m^2, significantly lower than at room temperature, with 130 kJ/m^2. This could either indicate lack of adaptation to low temperatures and/or the presence of metabolic processes active at room temperature protecting against UVB damage in *D. stevensoni*. The latter possibility could also explain why the estimated relative lethal UVB dose of *D. stevensoni* is similar to that of other non-marine ostracods where valves provide around 80% protection, despite the valves of *D. stevensoni* providing less protection. If such metabolic processes can repair UVB damage quickly, this may provide an alternative explanation why we could not quantify UVB damage in *D. stevensoni*.

KEY WORDS: *Darwinula stevensoni*, ancient asexual, DNA repair

INTRODUCTION

UVB (280-315 nm) radiation is one of the most important biological mutagens (Setlow et al., 1993; Rautio & Tartarotti, 2010), inducing the formation of cyclobutane pyrimidine dimers (CPDs) (Sancar & Tang, 1993; Rautio & Tartarotti, 2010), which inhibit DNA transcription and translation (Sancar & Tang, 1993). One way for organisms to remove these CPDs is excision repair (ER), which tends to be common across eukaryotes, but can be energetically costly if more than a single nucleotide requires repair (Sancar & Tang, 1993). Another important process of repair from UVB damage is enzymatic photoreactivation (Jagger & Stafford, 1965). The two enzymes involved in this process both use light energy; CPD-photolyase removes CPDs, while [6-4]-photolyase reverses pyrimidine-[6-4']-pyrimidine photoproducts (Friedberg et al., 1995).

Ostracoda or mussel-shrimps are small, bivalved Crustacea. Ostracods are very common in most surface waters, marine and non-marine, but they also occur in interstitial and even (semi-) terrestrial environments (Martens et al., 2008). These crustaceans are of great interest as a model group in ecological and evolutionary

research, because their calcified valves preserve well as microfossils, especially in lacustrine environments. Their excellent fossil record thus provides real-time frames for evolutionary processes (HOLMES & CHIVAS, 2002). Ostracods can also serve as proxies for climate (HORNE et al., 2012) and ecosystem changes because their fossilized valves allow reconstructing past climatic and environmental conditions (HOLMES & CHIVAS, 2002). In addition, non-marine ostracods are well-suited for investigations on the evolution of sex and parthenogenesis (MARTENS, 1998), because of their variety of reproductive modes. One of the ostracod families, Darwinulidae, reproduces exclusively asexually and is believed to have done so for at least 200 Myr (MARTENS et al., 2003); they thus represent one of the four examples of putative ancient asexuals in the animal kingdom. Karyological and allozyme studies have so far only found evidence for apomictic parthenogenetic reproduction in ostracods (BUTLIN et al., 1998; SCHÖN et al., 1998; SCHÖN & MARTENS, 2003).

SCHÖN et al. (2009) suggested that *D. stevensoni* is the most likely candidate to be a true ancient asexual. No reliable recent or fossil males have been found since at least 25 million years (STRAUB, 1952). Furthermore, this species appears to feature non-meiotic mechanisms such as gene conversion that could homogenize its genome (SCHÖN & MARTENS, 2003; SCHÖN et al., 2009) and possibly, highly efficient DNA repair (SCHÖN & MARTENS, 1998). Previous experiments with UVB (VAN DEN BROECKE et al., 2012) showed a strong correlation between the amount of UVB that is blocked by ostracod valves and the estimated relative lethal UVB doses. Certain ostracod valves blocked 80% and more of UVB radiation, thus providing effective shielding. Pigmented species from temporary habitats were best protected. These species also showed high estimated relative lethal UVB doses of 110 kJ/m^2 to 214 kJ/m^2. *Darwinulia stevensoni* was only protected against about 60% of UVB radiation by its valves, but the estimated relative lethal dose for this species was as high as for the other well-protected ostracods (130 kJ/m^2; VAN DEN BROECKE et al., 2012). These results may indicate that metabolic processes could also be involved in the repair of UVB damage in *D. stevensoni*.

Because of the lack of sufficient genomic data or any Expressed Sequence Tags, we used the following three techniques to quantify DNA repair in *D. stevensoni* after UVB exposure: the Polymerase Inhibition (PI) assay, Enzyme-Linked Immuno Sorbent Assay (ELISA) and dot blots. In an additional experiment, the influence of the temperature on UVB exposure and DNA damage was investigated. It has been suggested that UVB is a more important stressor at colder temperatures because enzymatic processes such as DNA repair mechanisms are slower at lower temperatures (HESSEN, 1996).

MATERIAL AND METHODS

Material

Darwinula stevensoni is common in all kinds of aquatic non-marine habitats, including lakes, rivers and interstitial habitats, freshwater to saline environments and arctic to (sub-) tropical conditions. All darwinulids are brooders and *D. stevensoni* has an average of 11–15 offspring in temperate regions (VAN DONINCK et al., 2003). Darwinulids generally have low fecundity as compared to other ostracods (GEIGER, 1998) and rather long life cycles of up to four years (MCGREGOR, 1969; RANTA, 1979). These features have so far made it impossible to establish synchronized long-term mass cultures as would be needed to test, for example, for maternal effects.

Darwinula stevensoni was collected from 'Hollandersgatkreek' (51° 16' 08'' N, 03° 32' 07'' E; Sint-Laureins, Belgium), where a monoclonal (as identified by the genetic markers COI and *Pgi*) population is known to occur in high densities throughout the whole year. All samples were randomly taken with a 200 µm mesh hand-net and subsequently stored in the laboratory for

acclimatisation as mass cultures in their habitat water at 15°C for one generation (because of the exceptionally long generation time, see above).

The experiments were performed using individuals that had been collected at various times during the course of the year. For the PI-experiments, individuals collected in spring, autumn and winter were used. For the ELISA-experiments, a pilot experiment investigated individuals from autumn, and for the subsequent experiment, ostracods from spring collections were used. For the dot blot experiments, individuals sampled in spring were screened in a pilot study, and samples collected in summer used for the more extensive study.

Methods

UVB exposure

To determine DNA damage as a response to UVB exposure, individual adult females of *Darwinula stevensoni* were randomly taken from the mass cultures. For all experiments with ostracods, six biological replicas were conducted in individual Petri dishes with EPA medium (96mg/L $NaHCO_3$, 60mg/L $CaSO_4.2H_2O$, 123mg/L $MgSO_4.7\ H_2O$, 4mg/L KCl and pH 7.4-7.8) on ice. The ostracods were exposed to UVB light from a 6W Vilber Loumat UV lamp (λ = 312 nm) with an intensity of 650 µW/cm^2 at a distance of approximately 15 cm to the lamp. UV intensity was measured with an UVB radiometer (UVP®). Exposure started with a dose of 1.95kJ/m^2 (= ±5 min), with a maximum of 140.4kJ/m^2 (= ±6 h) to ensure a large range of UV doses during exposures.

Polymerase Inhibition assay

The Polymerase Inhibition (PI) assay exploits the well-reported fact that the polymerase enzyme, which is routinely used in PCR reactions, stops replicating when it encounters a UV-induced adduct like a CPD in the template DNA (Jenkins et al., 2000). Consequently, the DNA segment that bears such damage provides a poor substrate for PCR. This will be reflected as a proportional reduction in the amount of amplified DNA from damaged templates as compared to non-damaged templates. On the other hand, DNA repair of the PCR target DNA segment should be measurable as a restoration in the amount of amplified template after exposure to UVB and subsequent repair.

After UVB exposure, the state of the *D. stevensoni* individuals (alive or dead) was recorded and DNA was extracted with the GeneReleaser standard protocol (Eurogentec). Subsequently, 5µl of DNA was used for PCR amplification.

The PI assay requires a fine-tuned optimisation, which was accomplished prior to testing for UVB damage. Three different genomic regions, an 850bp fragment of the single nuclear copy gene *hsp82*, 600bp of the nuclear multi-copy ITS region and 650bp of the mitochondrial COI gene were amplified by PCR to allow for comparisons of DNA repair and damage between nuclear and mitochondrial DNA and single or multi-copy regions. The following, species-specific primers for *D. stevensoni* were developed from existing sequences (Schön et al., 1998; Schön & Martens, 2003) (hsp82 FORW [TGACTACCTGGAGGAGAGGAA], hsp82 REV [CCAACATCCTCTATTTTTGGC], ITS FORW [TATCGTGAACCGTCTTGTCG], ITS REV [CGAGGTCCGACAGAAAGAAA], COI FORW [TACCTAATCTTAGGGGCCTGA], COI REV [AGGTGTTGGTATAGGATTGGG]).

For *hsp82*, an initial denaturation step at 95°C for 5 min was followed by 41 cycles of 15 s at 94°C, 1 min at 50°C and 1 min at 72°C. PCRs were performed in 25 µl volumes with PCR buffer (Tris·Cl, KCl, $(NH_4)_2\ SO_4$, 7.5 mM $MgSO_4$, bovine serum albumin, Triton® X-100, Factor SB; pH 8.7 (20°C), 1.5 mM dNTPs), 5 µl Q solution (Qiagen), 0.5 U Hifidelity Taq polymerase (Qiagen), 10 pmol of each primer, 7.5 µl RNAse free water and 5 µl of DNA template. The same conditions were used for the other two loci, except that the annealing

temperature for COI was 54°C and only 39 cycles were performed, while the annealing temperature for ITS was 52°C with 35 cycles. Electrophoresis of PCR products was conducted on 1.2 % agarose gels, which were stained with GelRed™ and photographed under UV light. The analyses of the relative intensity of PCR products as indicator for DNA damage were conducted with the program Image J (GIRISH & VIJAYALAKSHMI, 2004).

ELISA

In a follow-up on the PI-assay, we additionally performed ELISA experiments to detect the formation of CPDs. An added value of the ELISA technique is that the whole genome is investigated, employing a more direct detection technique using specific antibodies against CPDs. We conducted two different series of ELISA experiments, one pilot study in autumn and a more extensive study in spring. In the ELISA experiments, we exposed three different kinds of material to UVB: extracted DNA (4 replicates), living and dead *D. stevensoni* individuals (6 replicates each). If the bodies or valves of ostracods provide protection to UVB, extracted DNA should show the highest amount of UVB damage. If metabolic processes are involved in UVB protection, living ostracods should show lower UVB damage and more repair than dead ostracods. Thus, our hypothesis is that UVB damage is lowest in living individuals and is higher in dead ostracods, with the highest damage present in the DNA extractions. DNA was extracted from ostracods using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol. After UVB exposure and DNA extraction, DNA solutions in PBS were prepared with a concentration of 0.2 µg/mL following the protocol of MBL®. The DNA was coated to a microtiter plate covered with protamine sulfate. After overnight incubation, the specific Biotin-F(ab')2 fragment of anti-mouse IgG (H+L) (Zymed, Cat. No.62-6340) monoclonal antibody against CPDs (Cosmo Bio Co., Ltd) was distributed into the wells of the plate. After additional incubation, the first antibody was washed off and a second, enzyme-linked antibody was coated to the wells. As a final step, luminol (SUPERSIGNAL WEST FEMTO; Fisher Scientific) was added. Its light reaction indicates that the antigenes, in our case CPDs, are present, while the strength of the signal is proportional to the concentration of CPDs The strength of the luminol light reaction was measured with a VICTOR™ Light Luminescence Counter (PerkinElmer) and background levels were subtracted.

Dot blot

The third experimental approach included dot blot experiments, which have been successfully used to detect one type of CPDs (thymine dimers) in bacteria, phytoplankton and macroalgae (SINHA et al., 2001). Dot blotting is a simple technique being routinely used in laboratories to identify a known biomolecule in a biological sample. The ease and simplicity of the technique makes dot blotting an ideal diagnostic tool. In our experiments, dot blotting involved almost the same protocol as ELISA and thus the same specific monoclonal antibody against CPDs (Cosmo Bio Co., Ltd) was used. The most important difference to ELISA is the immobilization of DNA on a binding membrane, usually nitrocellulose or polyvinylidene fluoride, instead of a microtiterplate. DNA was extracted from ostracods using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol. For the dot blot, we mainly applied the protocol by SINHA et al. (2001) with some small modifications. After DNA extraction, we blotted 10ng DNA on an Amersham Hybond ECL Nitrocellulose Membrane (GE Healthcare). The visualization of the dot blot occurred with luminol, similarly to the ELISA experiment, but with a different variant (SUPERSIGNAL WEST PICO; Fisher Scientific) being less sensitive. Photos of the nitrocellulose membrane with the dots (Fig. 1) were developed on an Amersham Hyperfilm (GE Healthcare). Analyses of the intensity (darkness) of the dots on the membrane, and thus the relative amount of DNA damage, were conducted with the program Image J

(Girish & Vijayalakshmi, 2004). Animals for the pilot dot blot were sampled in spring, for the more extensive experiment in autumn.

Temperature-dependent experiment

All the UVB exposures for the PI assay, ELISA and dot blot experiments were conducted at room temperature. To examine the influence of the temperature on UVB damage, we also exposed 10 *D. stevensoni* individuals, placed in separated containers, to UVB at 4°C. At the same time, we placed 10 individuals at 4°C and 10 individuals at 25°C as controls without UVB exposure. The status of the animals was checked every 30 minutes.

Statistical analyses

All statistical analyses were conducted with STATISTICA. Data obtained from the different biological replicates were first tested for normality and homoscedasticity. If replicates for each treatment were normally distributed and did not differ in their variance, the average of the replicates was calculated per treatment and used for subsequent One-Way ANOVAs and, where applicable, for post-hoc Tukey HSD tests. Also, the correlation coefficient R and its statistical significance were calculated. For statistically comparing the estimated relative lethal doses of UVB at 4°C and room temperature, we conducted Chi Square tests.

RESULTS

Polymerase Inhibition assay

When the amount of PCR amplicons from living *D. stevensoni* (cultured at 15°C) is plotted against the intensity of UVB exposure, no clear pattern is observed for any of the three screened genetic regions (see Fig. 2 for the

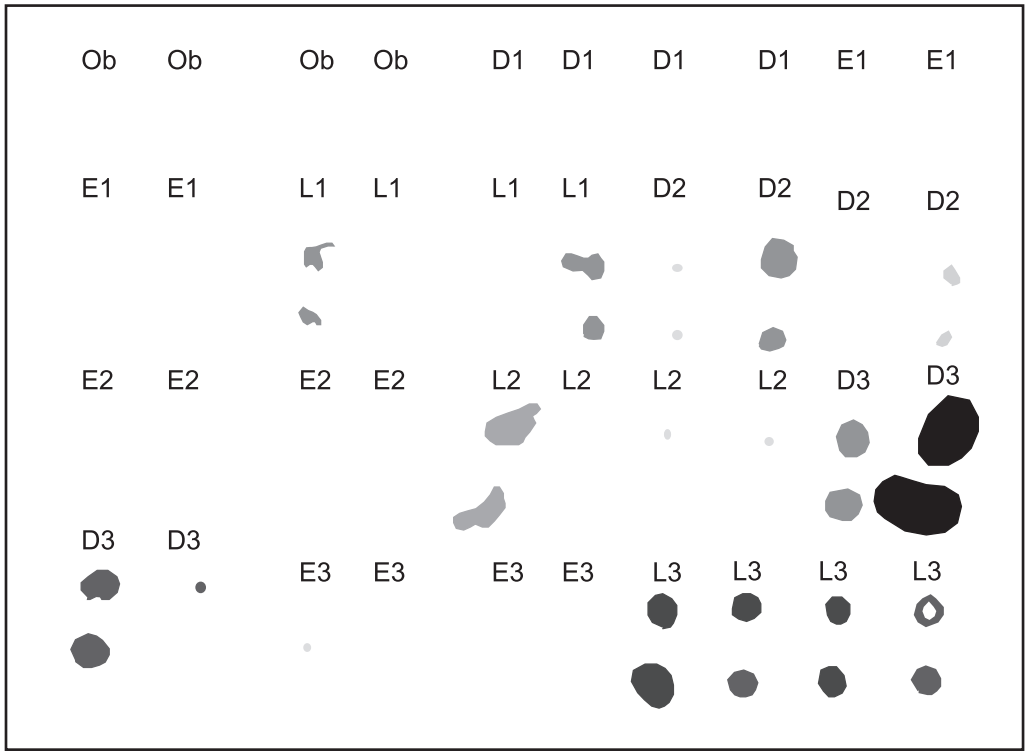

Fig. 1. – Dot blot experiment: photo of the dot blot membrane blotted with DNA from *Darwinula stevensoni*. The figure has been redrawn after the original photograph which is available online as supplementary material. Each dot represents one individual ostracod. The darkness of the dots corresponds to the amount of CPDs and thus the relative amount of DNA damage. Numbers correspond to hours of exposure: 1h (23kJ/m^2), 2h (47kJ/m^2) and 3h (70kJ/m^2). D = dead individuals; E = extracted DNA; L = living individuals; Ob = the positive controls.

example of the *hsp* gene). Data and figures for COI and ITS are not shown here because they are very similar to the results of *hsp* and also lack any clear patterns. They are available from the first author on request). Thus, the expected positive correlation of DNA damage to UVB dose is lacking. Furthermore, we observed high variability of relative DNA damage between the six replicas and lack of any difference between the nuclear and mitochondrial regions.

ELISA

In a preliminary ELISA pilot experiment, the three different types of material (alive, dead and DNA extractions), derived from ostracods collected in autumn, were each separately exposed to UVB. Under these conditions, DNA damage increased significantly with exposure duration (0kJ/m^2, 23kJ/m^2, 47kJ/m^2 and 70kJ/m^2) for the DNA extractions ($p=0.0016$, $H=14.47$, $df=15$) and the dead ostracods ($p=0.0012$, $H=18.07$, $df=15$). Post-hoc Tukey HSD tests revealed that UV damage in DNA extractions differed significantly between the three treatments groups ($p=0.0015$ to $p=0.0165$, $df=15$) while in dead ostracods, UV damage differed significantly between all four exposure times ($p=0.00015$ to $p=0.011$, $df=15$). We also found a positive correlation between the duration of exposure (UVB dose) and the relative amount of DNA damage in two of the three types of material ($R^2= 0.966$, $p<0.001$ for the DNA extractions and $R^2= 0.979$, $p<0.001$ for the dead ostracods). However, for the exposed living individuals, no significant difference in DNA damage was observed for different UVB doses and no significant correlation was found.

In a subsequent ELISA experiment (with animals sampled in spring), all three types of material were simultaneously exposed to UVB. This time, however, there were no significant differences or correlations between the different conditions (results not shown but available from the first author on request). Furthermore, the positive control (no UVB exposure) gave

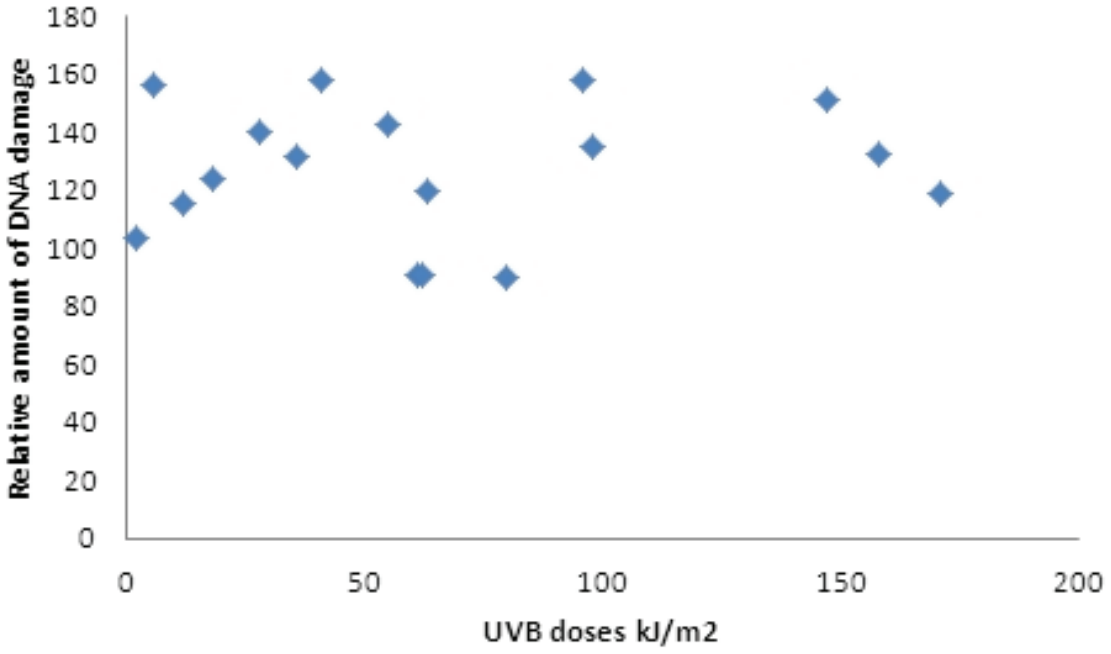

Fig. 2. – PI assay for part of the nuclear *hsp* gene of *Darwinula stevensoni*. The average, relative amount of DNA damage is plotted against the doses of UVB . The average, relative amount of DNA damage was calculated from the relative intensity of PCR products for all six replicas with the program Image J (Girish & Vijayalakshmi, 2004).

such high signals for UVB damage that it was no longer possible to subtract background levels. Also subsequent experiments with fresh products, new stocks of antibodies, and different DNA concentrations failed to show significant differences between the various treatments, and also did not give any clear correlation between the UVB doses and the amount of DNA damage.

Dot blot

As expected, this technique found no DNA damage in the positive controls not subjected to UVB exposure (Fig. 1). However, neither were CPDs detected from any DNA extractions, regardless of the UVB dose (Fig. 4). We also found no significant differences, with ONE-way ANOVAS, in the amount of DNA damage between dead or living ostracods having been exposed to three different UVB doses. With posthoc Tukey's HSD tests, however, a significant increase in DNA damage was found in both dead and living individuals depending on the duration of UVB exposure ($p=0.029$ and $p=0.003$ and $df=21$, respectively, for DNA damage in dead ostracods; $p=0.00016$ for both comparisons and $df=17$ for DNA damage in living ostracods, respectively). We also observed a positive correlation between DNA damage and UVB dose for the dead individuals, which was not statistically significant ($R^2= 0.960$, $p=0.09$). This first dot blot experiment was conducted with adult *D. stevensoni* collected in autumn. When we repeated the dot blot experiments with other ostracods in spring, we could not reproduce the results. Instead, we found high variability between replicas and failed to observe the expected positive correlation between DNA damage and UVB dose (results not shown but available from the first author on request).

Temperature-dependent experiment

After two hours of exposure at 4°C, all UVB-exposed ostracods were dead, while the unexposed ostracods were all still alive. Two hours of UVB exposure are equal to an UVB dose of 50kJ/m^2. Thus, the estimated relative lethal UVB dose for *D. stevensoni* at 4°C is at least 50kJ/m^2, while it was 130 kJ/m^2 at room temperature (Van den Broecke et al., 2012). This difference was statistically significant ($p<0.0001$; $df=9$, $Chi^2 = 429.31$).

DISCUSSION

With our PI assays, no clear correlation between UVB exposure and DNA damage was observed (Fig. 2), while Jenkins et al. (2000) did find such

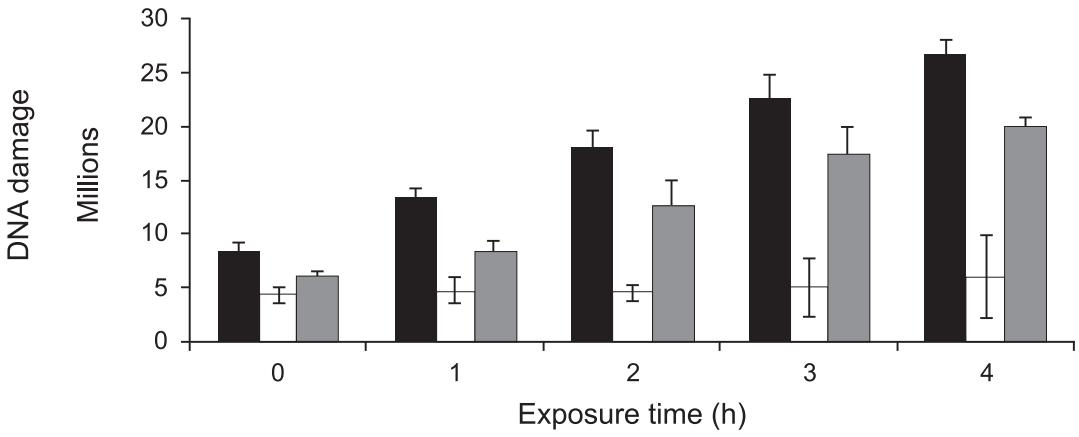

Fig. 3. – Pilot ELISA experiment for *Darwinula stevensoni* with DNA extractions (grey), dead (black) and living (white) ostracods being subsequently exposed to UVB. The numbers on the x axis are the hours of exposure [1h (23kJ/m^2), 2h (47kJ/m^2) and 3h (70kJ/m^2)], which are plotted against the average DNA damage and its standard deviation. Average DNA damage is calculated as the average number of CPDs from all replicas per treatment detected by VICTOR^{TM}.

a correlation when applying the same technique to mouse DNA. They investigated a larger PCR fragment of 1700bp although our three PCR regions together amount to 2100bp. The mouse DNA fragments were also much more sensitive to UVB damage as is illustrated by the maximal dose of 14kJ/m^2 that was used as compared to our maximal dose of 153kJ/m^2. Because only a limited region of the genome was analysed in our PI assays, chance might dictate whether the PCR target region of a few hundred basepairs is hit by UVB, and thus DNA damage is caused. This could also explain the large variability in relative DNA damage between the different replicas and genomic regions with the same UVB dose. It could also be that the response to UVB damage is too variable in the mixture of cells of which ostracods are composed as compared to the standardized animal cell cultures, which have been successfully used for PI assays (Govan et al., 1990; Kalinowski et al., 2000). Also, the PI-assay might be less suitable for investigating UV damage in living ostracods because of their high resistance to UVB. Ostracod valves block up to 80% of the UVB (Van den Broecke et al., 2012) and 60% in the case of *D. stevensoni*. It thus may be necessary to analyse larger parts of the genome to significantly increase the resolution power of the technique to quantify UVB damage.

Because of those concerns, we subsequently applied two other techniques, namely ELISA and dot blots, where DNA damage is detected in the entire genome. Because ostracods without valves die quickly, it was necessary to find other suitable material to test for the effect of ostracod valves in UVB protection (Van den Broecke et al., 2012). DNA extractions from *D. stevensoni*, which are not protected by valves, were exposed to UVB in the ELISA and dot blot experiments. We expected that UVB damage would be higher in DNA extractions than in living or dead ostracods. In the dot blot experiment, we found no CPDs in the extracted DNA and this could be due to the fact that the DNA was degraded. We also exposed dead individuals to UVB to test whether metabolic processes (Schön & Martens, 1998) might actively repair UVB damage in the DNA. In this case, we would expect more UVB damage in the dead ostracods than in the living ones. The preliminary results of the ELISA experiment, which we conducted in autumn, were promising. As expected, UVB damage increased with the UVB dose for all three conditions (Fig. 3). But when we repeated the experiment in spring exposing the three different kinds of material (DNA extractions, dead and living ostracods) simultaneously, the previous results could not be reproduced. Also the control without UVB exposure showed evidence for UVB damage and the previous, positive correlation between UVB dose and UVB damage was lacking. Furthermore, the various treatments showed high standard deviations. We tested for technical or contamination problems, but neither of these seems to be able to explain the inconsistency. Similarly for the dot blot experiments, the first results for the living and dead individuals followed our expectations (Fig. 4), but could not be repeated. This technique might have failed for the DNA extractions because DNA could have been degraded. For future experiments, it will be necessary to include positive controls to check for integrity of the DNA extractions used. As in the ELISA experiments, individuals for our different dot blot experiments were collected in different seasons (spring-summer). Studies on zooplankton have clearly demonstrated that the same species responds differently to UVB in different seasons (Strutzman, 1999; Tartarotti et al., 1999). Because of high intraspecific spatial and temporal variability, only results from the same lake and time should be compared when ranking species-specific UVB tolerances (Leech & Williamson, 2000). All individuals for our experiments came from the same water body but indeed, from different seasons. Because of the exceptionally long life cycle of *D. stevensoni* (1-4 years) and the low number of offspring per female (11-15 daughters) (Ranta, 1979; McGregor, 1969; Van Doninck et al., 2003), it has not yet been possible to establish laboratory cultures of *D. stevensoni* for UVB experiments. Such cultures may overcome possible seasonal and maternal

effects and should thus be established for similar experiments in the future.

An additional complicating factor in the investigation of UVB damage may be age differences of the exposed ostracods. Various studies have shown that adults of zooplankton tolerate UVB better than juvenile stages (Leeck & Williamson, 2000; Vega & Pizarro, 2000; Ramos-Jiliberto et al., 2004; Huebner et al., 2006). One of the few documented exceptions to this pattern is the higher adult mortality in the rotifer *Asplanchna girodi* compared with its juveniles (Grad et al., 2003). The life cycle of *Darwinula stevensoni* in Belgium as about one year (Van Doninck et al., 2003) and thus also in Hollandersgatkreek from where our material was collected, and up to 4 years in subarctic areas (Ranta, 1979). We used only one life stage, namely adults without embryos in our experiments, but since the individuals came from a natural population, we have no information on the actual age of the exposed ostracods. Van Doninck et al. (2003) and Ranta (1979) found that old and young adults coexisted during spring, which may have further contributed to the lack of reproducibility between our various ELISA and dot blot experiments. It is unfortunately not possible to derive the age of adult *D. stevensoni* from their body size or valve outlines. Therefore, it would probably be best to conduct future experiments in autumn, when only one age class of adult *D. stevensoni* is present. Furthermore, during such controlled sampling, the temperature and ambient UVB of the water body from which the ostracods are taken should also be measured.

The estimated relative lethal UVB dose for *D. stevensoni* was significantly larger at room temperature (130kJ/m^2; Van den Broecke et al., 2012) than at 4°C (50kJ/m$^{2)}$. In studies on a crab, *Cyrtograpsus* sp. (Moresino & Helbling, 2010), on *Daphnia catawba* and *Leptodiaptomus minutus* (Williamson et al., 2002), on *Daphnia pulicaria* (MacFadyen, 2004), and on *Evechinus chloroticus* and *Diadema setosum* (Lamare et al., 2006), mortality after UVB exposure was significantly higher at 15°C than at 20°C. These results all suggest that UVB tolerance is temperature-dependent because it involves enzymatic repair of UVB damage. Our result reflects either adaptation to repair at higher temperatures in *Darwinula stevensoni* and/or the presence of some kind of metabolic, enzymatic process providing protection against UVB damage. In the latter case this process would be active to a larger extent at room temperature than at 4°C as indicated by the significantly lower estimated relative lethal UVB dose at 4°C. Enzymatic repair of UVB damage is thus expected to be faster and more efficient at 20°C, which may explain why the estimated relative

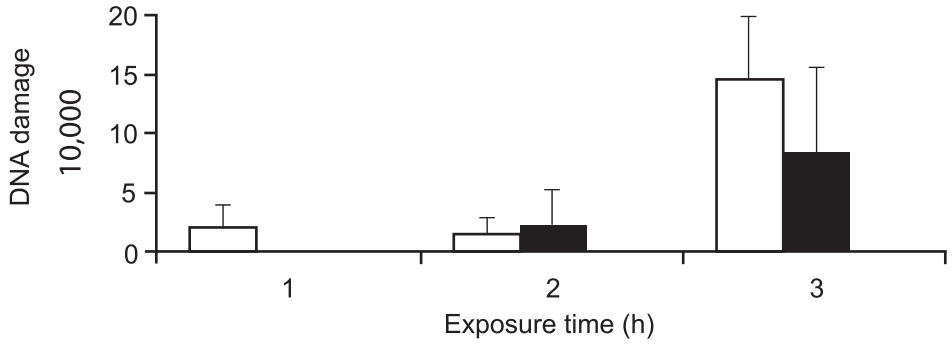

Fig. 4. – Pilot dot blot experiment for *Darwinula stevensoni* with DNA extractions (grey), dead (black) and living (white) ostracods. The numbers on the x-axis are hours of exposure [1h (23kJ/m^2), 2h (47kJ/m^2) and 3h (70kJ/m^2)], which are plotted against the average DNA damage and its standard deviation. Average DNA damage was calculated from the relative intensity of dots on the membrane from all replicas per treatment with the program Image J (Girish & Vijayalakshmi, 2004). Please note that we did not detect any DNA damage in the DNA extractions in the pilot dot blot experiment.

lethal doses of *D. stevensoni* at room temperature are similar to the estimated relative lethal doses of other non-marine ostracods despite the valves of *D. stevensoni* providing less protection (Van den Broecke et al., 2012). Our estimated relative lethal dose at 4°C could resemble the actual lethal dose of UVB for *D. stevensoni* without DNA repair or at suboptimal temperature conditions. Highly efficient and fast DNA repair during our exposure experiments is another plausible explanation for the variability of our results on the living individuals of *D. stevensoni* and for the absence of a positive correlation between DNA damage and duration of UVB exposure in living ostracods. In invertebrates, two important repair processes are known: nucleotide excision repair (NER, dark repair) and photo-enzymatic repair (PER, light repair) (Sancar, 1994a). NER is an energetically costly complex multi-protein, multi-step pathway, and is found in almost all taxa without being specific to UVB-induced DNA damage (Sinha & Häder, 2002). PER uses the enzyme photolyase and can reverse pyrimidine dimers (Sutherland, 1981; Mitchell & Karentz, 1993). Since it is a single-enzymatic process driven by photorepair radiation, it is less costly than NER (MacFadyen et al., 2004). Although PER is specific to UV-induced DNA damage, it is not present in all eukaryotic taxa investigated so far (Sancar, 1994b). PER and NER are also temperature-dependent mechanisms with more repair at higher temperatures (Williamson et al., 2002). Survival of UV-stressed *Daphnia* increased in the presence of PER (Siebeck & Böhm, 1991; Grad et al., 2001; Williamson et al., 2001, 2002; Huebner et al., 2006). A study by MacFadyen et al. (2004) provided additional evidence for PER in *Daphnia* at the molecular level. Other zooplankton taxa such as the rotifer *Asplanchna girodi* seem to utilise NER and have little to no PER (Sawada & Enesco, 1984; Grad et al., 2001). In juvenile *A. girodi*, however, evidence for PER has been found (Williamson et al., 2002; Grad et al., 2003). The importance of repair processes in copepods is not well understood and PER seems to be patchily distributed: both the cyclopoid copepod *Metacyclops mendocinus* and the calanoid copepod *Leptodiaptomus minutus* showed evidence for PER (Gonçalves et al., 2002; Williamson et al., 2002). Also Zagarese et al. (1997) found that PER accounted for the relatively high UVB tolerance in red *Boeckella gibbosa*, while little evidence of PER was found in *Boeckella gracilipes* (Zagarese et al., 1997; Tartarotti et al., 2000). Surprisingly, when exposing four different species of *Daphnia* to a single acute UVB dose, higher survival and repair rates were found at the lower experimental temperature (10°C compared to 20°C), indicating that the enhanced rate of PER at lower temperature contributed significantly to the recovery of these animals (Connelly et al., 2009). The same authors also confirmed that photorepair was the primary mechanism to remove DNA lesions in *Daphnia*. For *Darwinula stevensoni*, further research, for example using qPCRs, is necessary to identify which system of repair from UVB damage is active. Controls kept in the dark (thus preventing PER DNA repair) will be needed to test whether the lack of DNA damage is due to effective sunscreening in photorepair or to effective PER repair.

CONCLUSIONS

To conclude, there are many factors that must be considered when investigating the response to UVB in ostracods or other invertebrates. The valves are important in the protection against UVB (Van den Broecke et al., 2012) but also other factors such as seasonality (Strutzman, 1999; Tartarotti et al., 1999), age (e.g. Huebner et al., 2006) and temperature (Moresino & Helbling, 2010; Rautio & Tartarotti, 2010) of the habitat and during the experiment, are obviously of great importance and should be carefully controlled in future experimental set-ups for investigating UVB damage in living or dead ostracods. Future experiments could be further facilitated by using animals from lab cultures, which would overcome any possible maternal effects. Finally, repeating the experiments under a range of

different temperature conditions would help to determine whether the higher UVB damage in *D. stevensoni* at 4°C reflects a general adaptation of the species to higher temperatures or indicates the presence of temperature-dependent, metabolic repair processes.

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REFERENCES

Butlin RK, Schön I & Griffiths HI (1998). Introduction to reproductive modes. *In*: Martens K (ed), Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods. Backhuys Publishers, Leiden:1-24.

Connelly SJ, Moeller RE, Sanchez G & Mitchell DL (2009). Temperature effects on survival and DNA repair in four freshwater cladoceran *Daphnia* species exposed to UV radiation. Photochemistry and Photobiology, 85: 144-152.

Friedberg EC, Walker GC & Siede W (1995). DNA Repair and Mutagenesis. ASM Press, Washington DC.

Geiger W (1998). Population dynamics, life histories and reproductive modes. *In*: Martens K (ed), Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods. Backhuys Publishers, Leiden:215-228.

Girish V & Vijayalakshmi A (2004). Affordable image analysis using NIH Image/Image J. Indian Journal of Cancer, 41:47.

Gonçalves RJ, Villafañe VE & Helbling EW (2002). Photorepair activity and protective compounds in two freshwater zooplankton species (*Daphnia menucoensis* and *Metacyclops mendocInus*) from Patagonia, Argentina. Photochemical & Photobiological Sciences, 1:996-1000.

Govan HL, Valles-Ayoub Y & Braun J (1990). Fine-mapping of DNA damage and repair in specific genomic segments. Nucleic Acids Research, 18:3823-3830.

Grad G, Burnett BJ & Williamson CE (2003). UV damage and photoreactivation: timing and age are everything. Photochemistry and Photobiology, 78:225-227.

Grad, G, Williamson CE & Karapelou DM (2001). Zooplankton survival and reproduction responses to damaging UV radiation: a test of reciprocity and photoenzymatic repair. Limnology and Oceanography, 46:584-591.

Hessen DO (1996). Competitive trade-off strategies in Arctic *Daphnia* linked to melanism and UV-B stress. Polar Biology, 16:573-576.

Holmes J.A & Chivas A (2002). The Ostracoda: Applications in Quaternary Research. American Geophysical Union, Washington DC.

Horne d, Homes j, Finnberg v & Rodriquez-Lazaro j (2012) Ostracoda as Proxies for Quaternary Climate Change, Volume 17 (Developments in Quaternary Science).

Huebner JD, Young DLW, Loadman NL, Lentz VJ & Wiegand MD (2006). Age-dependent survival, reproduction and photorepair activity in *Daphnia magna* (Straus, 1820) after exposure to artificial ultraviolet radiation. Photochemistry and Photobiology, 82:1656-1661.

Hurtubise RD, Havel JE & Little EE (1998). The effects of ultraviolet-B radiation on freshwater invertebrates: experiments with a solar simulator. Limnology and Oceanography, 43:1082-1088.

Jagger J & Stafford RS (1965). Evidence for Two Mechanisms of Photoreactivation in *Escherichia coli* B. Biophysical Journal, 5:75-88.

Jenkins GJS, Burlinson B & Parry JM (2000). The Polymerase Inhibition Assay: a Methodology for the Identification of DNA-damage Agents. Molecular Carcinogenesis, 27:289-297.

Kalinowski DP, McCready SJ, Osman F & Yasui A (2000). Repair of UV damage in the fission yeast *Schizosaccharomyces pombe*. Mutation Research, 251:197-210.

Lamare MD, Barker MF, Lesser MP & Marshall C (2006). DNA photorepair in echinoid embryos: Effects of temperature on repair rate in Antarctic and non-Antarctic species. Journal of Experimental Biology, 209:5017-5028.

Leech DM & Williamson CE (2000). Is tolerance to ultraviolet radiation in zooplankton related to body size, taxon, or lake transparency? Journal of Applied Ecology, 10:1530-1540.

MacFadyen EJ, Williamson CE, Grad G, Lowery M, Jeffrey WH & Mitchell DL (2004). Molecular response to climate change: temperature dependence of UV- induced DNA damage and repair in the freshwater crustacean *Daphnia pulicaria*. Global Change Biology, 10: 408-416.

Martens K (1998). Sex and Parthenogenesis – Evolutionary Ecology of Reproductive Modes in Non-marine Ostracods. Backhuys Publishers, Leiden.

Martens K, Rossetti G & Horne DJ (2003). How ancient are ancient asexuals? Proceedings of the Royal Society B: Biological Sciences, 270:723-729.

Martens K, Schön I, Meisch C & Horne DJ (2008). Global diversity of ostracods (Ostracoda, Crustacea) in freshwater. Hydrobiologia, 595: 185-193.

McGRegor DL (1969). The reproductive potential, life history and parasitism of the freshwater ostracod *DarwInula stevensoni*. *In*: Neale JW (ed), The taxonomy, morphology and ecology of recent Ostracoda. Oliver & Boyd, Edinburgh: 194-221.

Mitchell DL & Karentz D (1993). The induction and repair of DNA photodamage in the environment. *In*: Young AR, Björn LO, Moan J & Nultsch W (eds), Environmental UV photobiology, Plenum Press:345-371.

Moresino RDH & Helbling EW (2010). Combined effects of UVR and temperature on the survival of crab larvae (Zoea I) from Patagonia: The role of UV-absorbing compounds. Marine Drugs, 8(5): 1681-1698.

Ramos-Jiliberto R, Dauelsberg P & Zúñiga LR (2004). Differential tolerance to ultraviolet-B light and photoenzymatic repair in cladocerans in a Chilean lake. Marine & Freshwater Research, 55:193-200.

Ranta E (1979). Population biology of *DarwInula stevensoni* (Crustacea, Ostracoda) in an oligotrophic lake. Annales Zoologici Fennici, 16:28-35.

Rautio M & Tartarotti B (2010). UV radiation and freshwater zooplankton: damage, protection and recovery. Freshwater Reviews, 3(2):105-131.

Sancar A (1994a). Mechanisms of DNA excision repair. Science, 266:1954-1956.

Sancar A (1994b). Structure and function of DNA photolyase. Biochemistry, 33:2-9.

Sancar A & Tang M (1993). Nucleotide excision repair. Photochemistry and Photobiology, 57: 905-921.

Sawada M & Enesco HE (1984). Effects of UV radiation on the lifespan of the rotifer *Asplanchna brightwelli*. Experimental Gerontology, 19:289-296.

Schön I & Martens K (1998). DNA repair in ancient asexuals – a new solution to an old problem? Journal of Natural History, 32:943-948.

Schön I & Martens K (2003). No slave to sex. Proceedings of the Royal Society B: Biological Sciences, 270:827-833.

Schön I, Rossetti G & Martens K (2009). Darwinulid ostracods: ancient scandals or scandalous gossip. *In*: Schön I, Martens K & van Dijk P (eds), Lost sex. Springer, The Netherlands:217-240.

Schön I, Butlin RK, Griffiths HI & Martens K (1998). Slow molecular evolution in an ancient asexual ostracod. Proceedings of the Royal Society B: Biological Sciences, 265:235-242.

Setlow RB, Grist E, Thompson K & Woodhead AD (1993). Wavelengths effective in induction of malignant melanoma. Proceedings of the National Academy of Sciences of the United States of America, 90:6666-6670.

Siebeck O & Böhm U (1991). UV-B effects on aquatic animals. Verhandlungen der Internationalen Vereinigung für theoretische und angewandte Limnologie, 24:2773-2777.

Sinha RP & Häder D-P (2002). UV-induced DNA damage and repair: a review. Photochemical & Photobiological Sciences, 1:225-236.

Sinha RP, Dautz M & Häder D-P (2001). A simple and efficient method for the quantitative analysis of thymine dimers in Cyanobacteria, Phytoplankton and Macroalgae. Acta Protozoologica, 40:187-195.

Straub EB (1952). Mikropaläontologische Untersuchungen im Tertiär zwischen Ehingen und Ulm a.d. Donau. Geologisches Jahrbuch, 66:433-523.

Strutzman PL (1999). A comparative study of ultraviolet radiation in different populations of *Diaptomus mInutes*. Journal of Plankton Research, 21:387-400.

SUTHERLAND BM (1981). Photoreactivation. Bioscience, 31:439-444.

TARTAROTTI B, CABRERA S, PSENNER R & SOMMARUGA R (1999). Survivorship of *Cyclops abyssorum tatricus* (Cyclopoida, Copepoda) and *Boeckella gracilipes* (Calanoida, Copepoda) under ambient levels of solar UVB radiation in two high-mountain lakes. Journal of Plankton Research, 21:549-560.

TARTAROTTI B, CRAVERO W & ZAGARESE HE (2000). Biological weighting function for the mortality of *Boeckella gracilipes* (Copepods, Crustacea) derived from experiments with natural solar radiation. Photochemistry and Photobiology, 73:314-319.

VAN DEN BROECKE L, MARTENS K, PIERI V & SCHÖN I (2012). Ostracod valves as efficient UV protection. Journal of Limnology, 71(1):119-124.

VAN DONINCK K, SCHÖN I, DE BRUYN L & MARTENS K (2002). A general purpose genotype in an ancient asexual. Oecologia, 132:205-212.

VAN DONINCK K, SCHÖN I, MARTENS K & GODDEERIS B (2003). The life-cycle of the ancient asexual ostracod *DarwInula stevensoni* (Brady & Robertson, 1870) (Crustacea, Ostracoda) in a temporate pond. Hydrobiologia, 500:331-340.

VEGA MP & PIZARRO R (2000). Lethal effect induced by ultraviolet-B in a planktonic copepod: role of the post-irradiation time on mortality measurements. Journal of Freshwater Ecology, 15:1-5.

WILLIAMSON CE, GRAD G, DE LANGE HJ, GILROY S & KARAPELOU DM (2002). Temperature-dependent ultraviolet responses in zooplankton: implications of climate change. Limnology and Oceanography, 47:1844-1848.

WILLIAMSON CE, NEALE PJ, GRAD G, DE LANGE HJ & HARGREAVES BR (2001). Beneficial and detrimental effects of UV on aquatic organisms: implications of spectral variation. Journal of Applied Ecology, 11:1843-1857.

ZAGARESE HE, FELDMAN M & WILLIAMSON CE (1997). UV-B induced damage and photoreactivation in three species of *Boeckella* (Copepods, Calanoida). Journal of Plankton Research, 19:357-367.

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Variability of the proximal phalanx in warmblood and coldblood horses – morphological and structural analyses

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ABSTRACT. Anatomical shape as well as bone geometry are important factors for the mechanical properties of stiffness and strength in bones. In the light of this statement, the primary aim of the study was to evaluate the variable forms of the proximal phalanx in different types of horses. Multivariate analyses of data from 81 horses revealed that the proximal phalanx has diverse spatial forms. Differences were observed particularly in the length of the bone and the breadth of its diaphysis. In horses with lighter morphotype, the phalanx is significantly narrower in its middle section. In the second stage of the study, geometrical parameters of the phalanx were analysed with the use of peripheral quantitative computed tomography (pQCT). Tomographic analysis was conducted at three levels: at 15%, 50% and 85% of the bone length. Based on the analysis, we concluded that most of the geometric parameters have higher values in coldbloods but only at the mid-diaphysis (50%) and at 85% of the bone length. Moreover, in coldblood horses, higher strength of the phalanx at these levels, expressed by Strength Strain Index, was observed. We did not observe any significant differences between warmblood and coldblood horses in the metaphyseal proximal region which is located at 15 % of the bone length.

KEY WORDS: bone, computed tomography, horses**,** proximal phalanx

INTRODUCTION

Diseases of limb bones in horses are one of the pathologies most commonly encountered in this species. Statistical data from many countries show that proximal phalanx fractures in the thoracic limbs are one of the most common injuries in horses (Yovich & McIlwraith, 1986; Ellis et al., 1987; Parkin et al., 2004; Dzierzęcka et al., 2006). On the basis of our four-year-long study conducted on 850 thoroughbred race horses, we concluded that diseases of the musculoskeletal system usually applied to segments of autopodium in thoracic limbs and were caused by injuries. Among the injuries, fractures comprised over 30% of all the registered cases and applied mostly to proximal phalanges (Dzierzęcka et al., 2006, 2007, 2008).

Proximal phalanges are usually subject to longitudinal and sagittal fractures (Yovich & McIlwraith, 1986; Ellis et al., 1987; Parkin et al., 2004, 2004; Powell, 2012). Such fractures are probably facilitated by the characteristic base shape at the proximal bone end. On the articular surface, there is an articular pit for articulation with the sagittal crest of the third metacarpal bone (in thoracic limb), or the third metatarsal bone (in pelvic limb). The crest "squeezes into" the articular foveola of the proximal phalanx acting like a splitting wedge, which often leads to this type of fracture (Dubs & Nemeth, 1975; Markel & Richardson, 1985; Ellis et al., 1987).

The mechanic endurance of the bone tissue, which determines its supporting functions, depends not only on the mineral composition

but also on its spatial architecture (Mow & Hades, 1991; Alho, 1993). In human medicine, densitometric methods are usually used for the evaluation of bone tissue quality. These methods are imperfect as they are unable to evaluate the spatial structure of the bone trabeculae. However, not only the mineralization level but also the trabecular architecture, which forms the bone tissue geometry, plays an important role in creating the tissue strength. In spatial constructions, durability is affected not only by the quality of the material but also by its proper architectural structure allowing for the distribution of the load on the bone (Mow & Hades, 1991; Czerwiński, 1994; Claes et al., 1995). The characteristics of macroarchitecture are most commonly shown by the use of osteometric techniques. However, it is the quantitative computed tomography (QCT) that delivers full information on the micro-architecture, i.e. geometric parameters of the investigated part of the skeleton (Charuta, et al. 2012; Dzierzęcka & Charuta, 2012).

Computer tomography is a non-invasive method of 3D imaging, which enables researchers to evaluate the densitometric and geometric parameters of the scanned bone fragment. Additionally, based on the assumptions made by Ferreti, the use of this method allows for determining the strain strength index (SSI). Ferreti assumed that bone durability depends on the properties of compact osseous tissue such as density and distribution on the circuit of the cross-section. The software calculates SSI based on tomographically-obtained volumetric bone mineral density (vBMD) and bone radius.

It is worth mentioning that the third metacarpal bone (MC3) has been a subject of numerous studies. Nevertheless, we focused our studies on the proximal phalanx because of the high frequency of fractures in this bone (Clegg, 2011; Ramzan & Palmer, 2011; Jacklin & Wright, 2012). Our analyses partially fill the gap in research in the field of osteological problems regarding this species. In horses, the mechanical properties of stiffness and strength in a bone also depend on the physical properties of the bone material, and the overall anatomical shape or bone geometry (Davies, 2002; Meulen et al., 2001; Radzki et al., 2006, 2007). As a result, we hypothesize that some horses may be subject to higher risk of fractures of the proximal phalanx related to its less favorable anatomical shape. For that reason, it may be informative to compare the spatial form of this bone in horses of different breeds. We can assume that the spatial form will be different in warmblood horses and in coldblood horses due to different morphotypes they represent (Komosa et al., 2006; Brooks et al., 2010). The proximal phalanx may have a variable shape in cross-bred horses as their phenotype is not uniform. Some may be more osteologically-related to warmbloods or to coldbloods.

As a result of the above hypothesis, the primary aim of this study was to determine the shape of the proximal phalanx in warmblood, coldblood and cross-bred horses as an intermediate type. This part of our study included osteometry involving the use of multivariate statistical methods.

The secondary goal of this study was to show the diversity of geometrical parameters of bones with the use of peripheral quantitative computer tomography (pQCT) and compare the SSI values in warmblood and coldblood horses as extremely diverse types of horses.

MATERIAL AND METHODS

The study was conducted on 81 horses destined for slaughter for reasons unrelated to this research. Among them were 33 Polish Halfbred Horses. This is a warmblood X thoroughbred used for sports purposes. Their height at the withers ranges between 165 - 174 cm, and their body weight between 540 - 620 kg. The second group comprised 30 coldblood horses. These were Polish Coldblood Horses, which are mainly used as draft animals. They are shorter, standing 148-160 cm . Their mass, however, is higher; between 600 - 800 kg. Individuals weighing over 900 kg can also occur. The remaining 18

horses were cross-breds with intermediate characteristics compared to the other two types. The animals were aged between 3 and 15 years.

In the first stage of the study, the proximal phalanx of the left thoracic limb was dissected and measured in all the horses. Using the standard method by DRIESCH (1976), five measurements were performed on every preparation. The following parameters of the phalanx were analysed:

1. Greatest length (GL)
2. Smallest breadth of the diaphysis (SD)
3. Breadth of the proximal end (Bp)
4. Breadth of the distal end (Bd)
5. Depth of the proximal end (Dp)

These metric features comprised benchmark data for further multivariate statistical analyses. Firstly, an exploratory method - Principal Components Analysis - was used to determine similarities in the phalanx between the three groups of horses. The second method applied was the Discriminant Analysis, which indicated those features that were most efficient in differentiating the phalanx in the studied horses. Next, based on the obtained parameters, a size index (expressed as a percentage) was developed with regard to the proximal phalanx. Statistical analyses were performed using the Statistica v 9.0 software.

The second stage of the study involved the comparison of geometrical parameters at three levels (15%, 50% and 85%) of the pastern bone in warmbloods and coldbloods. The length of every analysed bone was measured with a digital slide caliper at three locations. The obtained values were then input into the CT software. After initial scanning, it was possible to establish the referential line that was tangent to the articular surfaces. Since the bone length was already input, adding the measuring lines on any given bone area had to be performed next. In the case of our bones, the lines were drawn at 15%, 50% and 85% of the bone length (Fig. 1). The thickness of the analyzed slices was 0.07 mm.

However, at this stage of the study, the number of horses was limited to 20. Ten phalanges of warmblood horses and ten of coldblood horses were randomly selected. Cross-breds were not included. Peripheral Quantitative Computed Tomography (pQCT) with XCT Research SA Plus (Stratec Medizintechnik GmbH, Pforzheim Germany) was used to analyse the structure of the bones according to Ferretti's method (FERRETTI et al., 1995, 1996).

In the analysed phalanges, the following geometrical parameters were determined: total bone area (TOT_A) mm^2, trabecular area (TRAB_A) mm^2, cortical thickness (CRT_THK_C) mm, periosteal circumference (PERI C) mm, endocortical circumference (ENDO_C) mm and Strength Strain Index (SSI = RP_CM_W) mm^3. The analysis was performed with the voxel size of 0.07 mm and scanning speed of 4 mm/min. The area for the analysis was determined after preliminary scanning (20 mm/s). Threshold coefficient, differentiating compact bone from

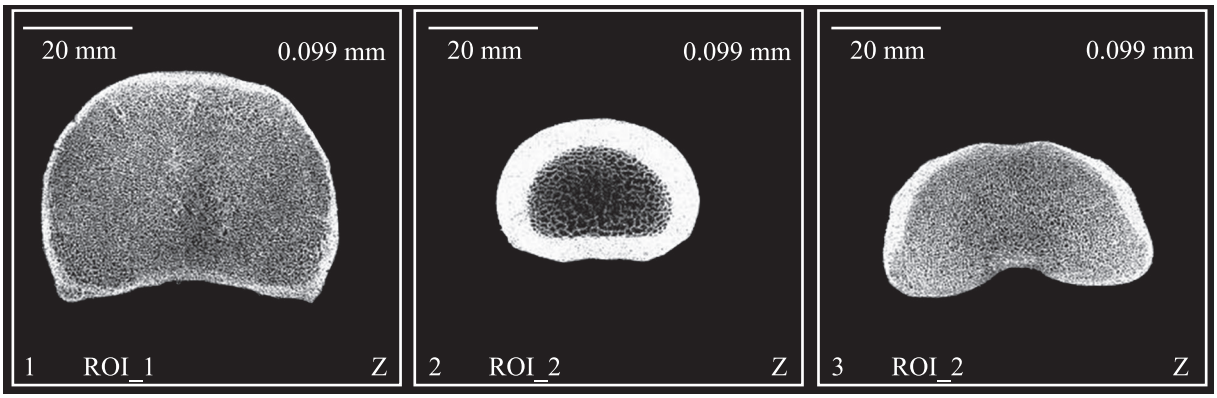

Fig. 1. – The view of three measurements of proximal phalanx (successively 15, 50 and 85% of the bone length) from the computed tomography image.

TABLE 1

Basic statistics of the proximal phalanx parameters in the analysed groups of horses.

Variable	N	Mean value (cm)	Minimum (cm)	Maximum (cm)	Standard deviation
Warmblood horses					
Greatest length	33	10.3	8.9	11.7	0.7
Smallest breadth of the diaphysis	33	4.0	3.1	5.0	0.4
Breadth of the proximal end	33	6.4	5.1	7.6	0.5
Breadth of the distal end	33	5.5	4.5	6.5	0.4
Depth of the proximal end	33	4.3	3.5	5.1	0.4
Coldblood horses					
Greatest length	30	9.7	8.6	10.4	0.4
Smallest breadth of the diaphysis	30	4.5	3.9	4.9	0.2
Breadth of the proximal end	30	6.8	5.6	7.8	0.5
Breadth of the distal end	30	5.7	4.7	6.6	0.4
Depth of the proximal end	30	4.3	3.7	4.9	0.3
Cross-bred horses					
Greatest length	18	9.7	9.1	10.2	0.3
Smallest breadth of the diaphysis	18	4.2	3.6	4.8	0.3
Breadth of the proximal end	18	6.6	6.0	7.1	0.3
Breadth of the distal end	18	5.5	5.1	6.3	0.3
Depth of the proximal end	18	4.4	4.0	4.9	0.2

trabecular bone, was determined at the level of 0.900 cm^{-1}.

RESULTS

Multivariate analyses of the proximal phalanx

I.1. Basic statistics and Principal Components Analysis.

The first stage of the study involved basic statistical analysis of all the metrical features of the proximal phalanx. The analysis was performed separately for warmblood, coldblood and cross-bred horses (Table 1). These parameters were compared between groups of horses with the use of one-way ANOVA. Average values of GL and SD are highly significantly different ($p \leq 0.001$), while Bp is significantly different ($p \leq 0.05$). No statistical differences were observed between the average values of Bd and Dp.

The next stage was to present the morphometric differences of the proximal phalanx between the horse types mentioned. Principal Components Analysis was used for that purpose. The idea of the method is to display and describe the variability of the phalanx based on all its

TABLE 2

Correlations between metric features and principal components after varimax rotation. Load values in bold show strong correlation with a given component.

Variable	Principal Component 1	Principal Component 2
Greatest length	0.14	**0.96**
Smallest breadth of the diaphysis	**0.94**	0.03
Breadth of the proximal end	**0.90**	0.31
Breadth of the distal end	**0.89**	0.34
Depth of the proximal end	0.65	0.58

measurements altogether. Moreover, the analysis revealed new, so-called hidden factors, also referred to as components. From among the principal components selected, the highest two jointly explain 87.8% of the total variance determined by all the primary variables, i.e. measurements of the phalanx. The result is very high. Therefore, the two-dimensional graph based on the two components is highly representative of the proximal phalanx variability in the studied horses (Fig. 2).

Following a varimax rotation, each measurement was ascribed a load that expressed its correlation with a given principal component (Table 2). Principal component 1 is highly correlated with all the parameters of the proximal phalanx that describe its breadth. Thus, it can be called a "breadth component". Principal component 2, on the other hand, is a "length component" because the feature it correlates with to the highest extent is GL. The two-dimensional diagram clearly showed the variability of the proximal phalanx between the warmblood and coldblood horses. In most cases, however, cross-

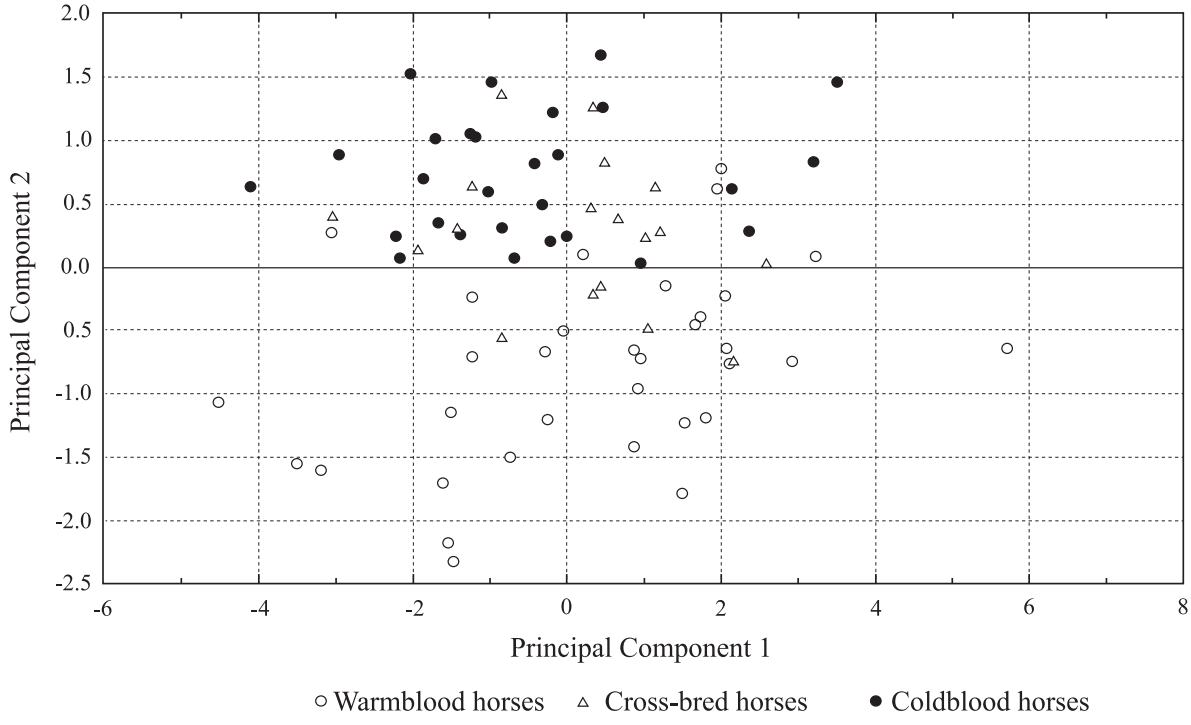

Fig. 2. – Two-dimensional graph showing two strongest principal components.

TABLE 3

Results of the Canonical Discriminant Analysis.

Variable N=81	Wilks' Λ	Partial Wilks' Λ	F value (2.76)	Level of significance
Smallest breadth of the diaphysis	0.692	0.502	37.674	**<0.001**
Greatest length	0.542	0.641	21.240	**<0.001**
Depth of the proximal end	0.374	0.930	2,844	0.064
Outside the model:				
Breadth of the proximal end	0.339	0.976	0.924	0.401
Breadth of the distal end	0.343	0.988	0.458	0.634

breds resemble coldblood horses rather than warmblood ones as regards the shape of the bone.

I.2. Canonical Discriminant Analysis

The aim of the Canonical Discriminant Analysis was to identify those metrical features that had the highest influence on the classification of a given bone to one of the three groups of horses. The stepwise progressive analysis model was chosen. Apart from the F test, the discriminant analysis uses a parameter called Wilk's Lambdas (Λ). This parameter describes the statistical significance of the model's discriminant power following the introduction of a given metric feature. Partial Wilk's Lambdas, on the other hand, shows the input of a metric feature into the discrimination between the groups. Decrease of this value indicates that the input of the metric feature increases.

The discriminant analysis demonstrated that three out of five features determine the classification of the phalanx. However, only two of them were statistically significant - smallest

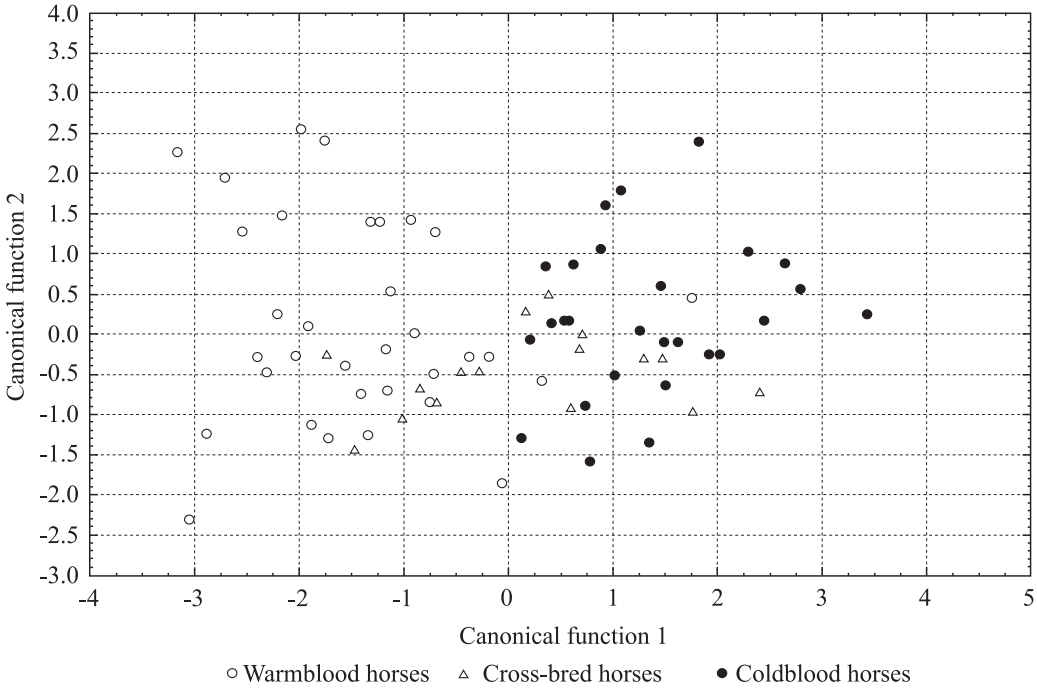

Fig. 3. – Two-dimensional graph showing canonical discriminant functions.

TABLE 4

Results of one way variance analysis for the proximal phalanx index in different types of horses.

Source	DF	SS	MS	F	P
Between groups	2	844.9	422.4	58.06	0.000
Within groups	78	567.5	7.3		
Total	80	1412.4			

breadth of the diaphysis (SD) and greatest length (GL) (Table 3).

The model did not include the parameters representing the breadth of the proximal and distal ends. Canonical discriminant functions allowed us to present once again the variability of the distal phalanx on the two-dimensional graph (Fig. 3).

I.3. Proximal phalanx index

Two most significant metric features of the phalanx were used to create a percentage index of its shape. The following formula was used: SD/GL x 100%. The mean value of the index was 38.91 %, 43.15 % and 46.21 % for the warmblood, cross-bred and coldblood horses respectively. One-way ANOVA conducted for the index showed that mean values for the groups mentioned above differ very significantly (Table 4).

Next, a *post hoc* procedure, Fisher's Least Significant Difference test, was conducted. It showed that the means for each group were significantly different from one another. The critical p-value was $p \leq 0.001$. Fig. 4 presents the examples of varied shapes of the distal phalanx in different types of horses.

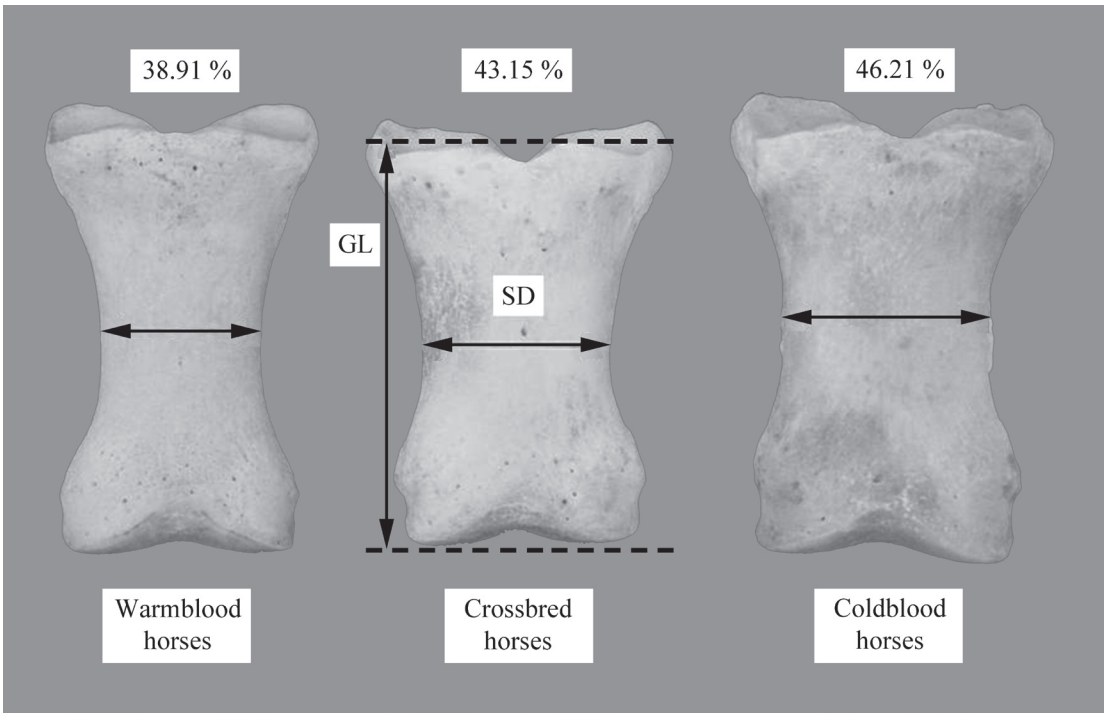

Fig. 4. – Shapes of the proximal phalanx and values of its index for Warmblood, Cross-bred and Coldblood horses.

TABLE 5

The value of T– test, testing the difference between geometric parameters of the pastern bones of the Warmblood horses and Coldblood horses at 15%, 50% and 85% of its length.

Variable	Meanvalue Warmbloodhorses	Meanvalue Coldbloodhorses	T value	Level of significance
at 15% of its length				
Total bone area	2145.13	2001.36	1.079	0.295
Trabecular area	965.30	900.30	1.088	0.291
Cortical thickness	4.54	5.41	-1.792	0.090
Periosteal circumference	163.72	158.33	1.060	0.303
Endocortical circumference	135.22	124.33	1.973	0.064
Strength strain index	14121.05	15632.07	-0.818	0.424
at 50% of its length				
Total bone area	969.68	1244.20	-2.412	**0.027**
Trabecular area	436.15	559.63	-2.416	**0.026**
Cortical thickness	6.64	6.23	0.985	0.337
Periosteal circumference	109.88	124.31	-2.523	**0.021**
Endocortical circumference	68.16	85.16	-2.653	**0.016**
Strength strain index	6572.25	9484.25	-2.316	**0.032**
at 85% of its length				
Total bone area	1117.02	1370.91	-2.837	**0.011**
Trabecular area	502.09	616.56	-2.848	**0.011**
Cortical thickness	5.90	5.48	1.026	0.318
Periosteal circumference	117.99	131.00	-2.888	**0.010**
Endocortical circumference	80.88	96.54	-2.905	**0.009**
Strength strain index	8301.89	10434.70	-2.196	**0.041**

Comparison of geometrical parameters of the proximal phalanx between the warmblood and coldblood horses

The number of horses to undergo computed tomography was limited to 20 compared with the initial group. Ten warmblood and ten coldblood horses were randomly selected for the analysis. T-test was used to compare all the geometrical parameters between the two groups (Table 5).

The test showed that at 15% of the bone length, phalanges in both groups of horses did not present any significant differences regarding the geometrical parameters analysed. However, at the mid-diaphysis and at 85% of its length, the bones in warmblood and coldblood horses differ significantly with respect to all the parameters but one - cortical thickness (CRT_THK_C). Parameters that differed, i.e. TOT_A, TRAB_A, PERI_C, ENDO_C and SSI,

were higher in coldblood horses. As for CRT_THK_C, it reached different values compared with other parameters. It was the highest at the mid-diaphysis and the lowest at 15 % of the bone length. It is an opposite relation or a mirror image, so to say, compared with the values of the other parameters (Table 5).

DISCUSSION

The present study indicates that the proximal phalanx has a significantly different shape in different groups of horses. The index we created compares the breadth of the diaphysis (SD) with the greatest length of the phalanx (GL). Since the index is expressed as a percentage, the meaning of a low value is that the shape of the bone resembles an hourglass. The diaphysis is the narrowest in warmblood horses, medium in cross-breds and the broadest in coldblood horses. Slim phalanx in warmbloods stems both from the increase of its total length as well as narrower diaphysis. Multivariate analyses, especially the discriminant analysis, indicated that the breadth of the phalangeal diaphysis and the length of the bone play the most important role in classifying the animal as a given morphological type. It is interesting in terms of the phalanx shape that cross-bred horses are more similar to coldbloods than to warmbloods.

Our results coincide with those obtained by Hildebrand (1987), Biewener (1991) and Pieszka et al. (2011), who demonstrated similar architecture of the third metacarpal bone, which is a part of the manus of the thoracic limb. Manus bones undergo temporary deformations when the horse gallops. Therefore, resistance to bending, which also takes place when a horse lands after a jump, is particularly important. In coldblood horses, on the other hand, diaphyses in limbs can be compared to columns, adapted to resist compressive forces, whose width is equal along the entire length (Bartosik, 1957). Compressive forces are related to high body mass of these horses. Physical activity exerts lower influence. In other words, the limbs of coldbloods are not exposed to such overloads as in saddle horses (galloping, jumping). Nevertheless they have to permanently withstand the vertical pressure of higher body mass. The body mass of a coldblood horse with height at the withers of approximately 160 cm is approximately 800 kg or more while a warmblood horse with the same height weighs only 500 kg. A similar problem was exposed by Nauwelaerts et al. 2011 when studying the inertial properties of separate sections of limbs. These researchers showed that the ratio between the total body mass and the foreleg pastern mass (body part composed of proximal and middle phalanx) is similar in horses of different morphotypes. We think that this conclusion confirms our belief because it suggests that higher pastern mass is obtained by increased breadth and circuit of the proximal phalanx. The deep digital flexor tendon and other accompanying anatomical structures are also broader. As far as the length of the pastern as a body part is concerned, it can be significantly different even within horses of the same breed or between related breeds (Komosa & Purzyc, 2009; Sobczuk & Komosa, 2012). Such diversity of shape among pasterns and bones that compose them confirms the need of conducting such anatomical analyses.

Moreover, in the context of bone strength, densitometric parameters should be considered as well as structural features, which are also referred to as architectural features (Ferretti et al., 1995, 1996; Rauch & Schoenau, 2001). In our study, structural features of the phalanx were determined by the evaluation of the following geometrical parameters: total bone area, trabecular area, cortical thickness, periosteal circumference and endocortical circumference. Our study is particularly valuable because the pQCT technique used allowed us to conduct an integrated evaluation of both material and structural features of the bone tissue through the determination of the Strength Strain Index.

The first important observation resulting from our study was that bone strength at 50% of the diaphysis was significantly higher in coldblood

horses, whose total bone area and trabecular area measured in this region were significantly higher. Similarly, the values of periosteal circumference and endocortical circumference measured at the mid-diaphysis of the phalanx were also higher in coldblood horses. As in the case of the mid-diaphysis, bone strength and thus, total bone area, trabecular area, periosteal circumference and endocortical circumference, were significantly higher in coldblood horses.

Similar observations regarding long bones were made by other researchers, who compared geometrical parameters of the radius and tibia in trained and untrained horses. When the geometrical parameters of bones increased, as occurred in the trained horses, bone strength expressed as the Strength Strain Index grew as well (NICHOLSON & FIRTH, 2010). Larger diameter of the bones, i.e. more peripheral location of the cortical substance, increases bone resistance to bending and twisting forces. Therefore, the bone whose cortical substance constitutes the farthest periphery, i.e. whose section is close to a circle, is stronger than other bones with the same cross-sectional area (KHAN et al., 2001). In our study, the location of the compact bone was determined using periosteal circumference and endocortical circumference. The results indicate that the phalanges in coldblood horses are more resistant to forces both at the mid-diaphysis and at 85% of the bone length. In the areas discussed, the diameter of the pastern bone is close to a circle.

At the same time, our study did not show any significant differences in cortical thickness between warmblood and coldblood horses in any of the measurement areas, nor did the studies on the radius and tibia in trained and untrained horses (NICHOLSON & FIRTH, 2010).

Our study, together with the results of research conducted on radius and tibia by NICHOLSON & FIRTH (2010) and on adult rats by JEE et al. (1991), confirms that varying impact of different types of genetic strain induces the increase of periosteal circumference, endocortical circumference, total bone area and trebecular area in the bones. However, it does not lead to the increase of cortical thickness (FIRTH et al., 2005; VERHEYEN et al., 2006; NICHOLSON & FIRTH, 2010). The differences in cortical thickness in the three measurement areas are particularly noticeable in the warmblood horses. In these horses, the parameter in question is, on average, even 30% higher at the mid-diaphysis than at 15% of the phalanx length. From the anatomical perspective, cortical thickness corresponds to the substantia compacta, which builds the bone at its circumference. The increase in thickness of this layer inside the phalangeal diaphysis is accompanied by a simultaneous decrease of diaphyseal breadth (SD), which occurs mostly in warmblood hoses. The correlation between the shape of the bone and its geometry visible on computed tomography can be related to the adaptation of the proximal phalanx to deformations that take place when warmblood horses gallop.

REFERENCES

ALHO A (1993). Mineral and mechanics of bone fragility fractures. Acta Orthop., 64:227-232.

BARTOSIK A (1957). The structure of metacarpal bones of some breeds and types of horses (*in Polish:* Struktura kości nadpęcia koni niektórych ras i typów użytkowych). Rocz. Nauk Roln., 71: 155-209.

BIEWENER AA (1991). Musculoskeletal design in relation to body size. J. Biomech., 24:19-29.

BROOKS SA, MAKVANDI-NEJAD S, CHU E, ALLEN JJ, STREETER C, GU E, MCCLERRY B, MURPHY BA, BELLONE R & SUTTER NB (2010). Morphological variation in the horse: defining complex traits of body size and shape. Anim. Genet., 41:159-165.

CHARUTA A, DZIERZĘCKA M & BIESIADA – DRZAZGA B (2012). Evaluation of densitometric and geometric parameters of tibiotarsal bones in turkeys. Bull. Vet. Inst. Pulawy., 56:379 - 384.

CLAES LE, WILKE HJ & KIEFER H (1995). Osteonal structure better predicts tensile strength of healing bone than volume fraction. J. Biomech., 28:1377-1390.

Clegg PD (2011). Musculoskeletal disease and injury, now and in the future. Part 1: fractures and fatalities. Equine Vet. J,. 43:643-649.

Czerwinski E (1994). Quantitative evaluation of changes occurring in the spongious cortical bone under the influence of fluor and their diagnostic meaning. Collegium Medicum UJ, Kraków.

Davies HMS (2002). Dorsal metacarpal cortex ultrasound speed and bone size and shape. Equine Exercise Physiology 6 in Equine Vet. J. Suppl., 34:337-339.

Driesch A (1976). A guide to the measurements of animal bones from archaeological sites. Peabody Museum Bulletin, 1:1-136.

Dubs B & Nemeth F (1975). Therapie und Prognose der Fesselbeinfrakturen. Schweizer Archiv für Tierheilkunde, 117:299-309.

Dzierzęcka M & Charuta A (2012). The analysis of densitometric and geometric parameters of bilateral proximal phalanges in horses with the use of peripheral quantitative computed tomography. Acta Vet. Scand., 54 41-51.

Dzierzęcka M, Charuta A, Wąsowski AJ, Bartyzel BJ & Janiuk I (2008). Injuries of limb joints during race training of 2-year-old Thoroughbred horses. Bull. Vet. Inst. Pulawy., 52:175-178.

Dzierzęcka M, Wąsowski AJ & Kobryń H (2007). The leg bones injuries during race training in two–year old thoroughbred horses. Med. Wet., 63:574-577.

Dzierzęcka M, Wąsowski AJ, Skubiszewska D, Bartyzel BJ & Karbowicz M (2006). Injuries of limb structures of locomotion apparatus in two – year old thoroughbreds being in racing training. Veterinarija ir Zootechnika, 35:41-43.

Ellis DR, Simpson DJ, Greenwood RE & Crowchurst JS (1987). Observations and management of fractures of the proximal phalanx in young Thoroughbreds. Equine Vet. J., 19:43-49.

Ferretti JL, Capozza RF & Zanchetta JR (1996). Mechanical validation of a tomographic (pQCT) index for noninvasive estimation of rat femur bending strength. Bone, 18:97-102.

Ferretti JL, Gaffuri O, Capozza RF, Cointry G, Bozzini C, Olivera M, Zanchetta JR & Bozzini CE (1995). Dexamethasone effects on mechanical, geometric and densitometric properties of rat femur diaphyses as described by peripheral quantitative computerized tomography and bending tests. Bone, 16:119-124.

Firth EC, Rogers CW, Double M & Jopson NB (2005). Musculoskeletal responses of 2-year-old Thoroughbred horses to early training. Bone parameters in the third metacarpal and third metatarsal bones. N. Z. Vet. J., 53:101-112.

Hildebrand M (1987). The mechanics of horse legs. Amer. Scient., 75:594-601.

Jacklin BD & Wright IM (2012). Frequency distributions of 174 fractures of the distal condyles of the third metacarpal and metatarsal bones in 167 Thoroughbred racehorses (1999-2009). Equine Vet. J., 44:707-713.

Jee WSS, Li XJ & Schaffer MB (1991). Adaptation of diaphyseal structure with aging and increased mechanical usage in the adult rat: A histomorphometrical and biomechanical study. Anat. Rec., 230:332-338.

Khan K, McKay H, Kannus P, Bailey D, Wark J & Bennell K (2001). Physical activity and bone health. Human Kinetics, Illinois, USA.

Komosa M, Moliński K & Godynicki S (2006). The variability of cranial morphology in modern horses. Zoolog. Sci., 23:289-298.

Komosa M & Purzyc H (2009). Konik and Hucul horses: A comparative study of exterior measurements. J. Anim. Sci., 87:2245-2254.

Markel MD & Richardson DW (1985). Noncominuted fractures of the proxy mal phalanx in 69 horses. JAVMA, 186:573.

Meulen MCH, Jepsen KJ & Mikic B (2001). Understanding bone strength: size isn't everything. Bone, 29:101-104.

Mow VC, Hades WC (1991). Basic orthopedics biomechanics. Raven Press, New York.

Nauwelaerts S, Allen WA, Lane JM & Clayton HM (2011). Inertial properties of equine limb segments. J. Anat., 218:500-509.

Nicholson CL & Firth EC (2010). Assessment of bone response to conditioning exercise in the radius and tibia of young thoroughbred horses using pQCT. Journal of Musculoskeletal and Neuronal Interactions, 10:199-206.

Parkin TD, Clegg PD, French NP, Proudman CJ, Riggs CM, Singer ER, Webbon PM & Morgan KL (2004). Risk of fatal distal limb fractures among Thoroughbreds involved in the five types of racing in the United Kingdom. Vet. Rec., 154:493-497.

Pieszka M, Łuszczyński J & Baranowska A (2011). Frequency of limb injures between thoroughbred and Arabian horses. Iran. J. Applied Anim. Sci., 1:69-71.

Powell SE (2012). Low-field standing magnetic resonance imaging findings of the metacarpo/metatarsophalangeal joint of racing Thoroughbreds with lameness localised to the region: a retrospective study of 131 horses. Equine Veterinary Journal, 44:169-177

Radzki RP, Bieńko M, Kapica M, Puzio I, Dobrowolski P & Filip R (2006). Effect of β_2-adrenergic receptor agonist clenbuterol on the mineralization of tibiae in rats with established osteopenia. Med. Wet., 62:824-826.

Radzki RP, Bieńko M & Filip R (2007). Influence of strontium ranelate on mineral and mechanical properties of the femur in orchidectomized rats. Med. Wet., 63:1630-1634.

Ramzan PH & Palmer L (2011). Musculoskeletal injuries in Thoroughbred racehorses: a study of three large training yards in Newmarket, UK (2005-2007). Veterinary Journal, 187:325-329.

Rauch F & Schoenau E (2001). The developing bone: slave or master of its cells and molecules? Pediatr. Res., 50:309-314.

Sobczuk D & Komosa M (2012). Morphological differentiation of Polish Arabian horses - multivariate analysis. Bull. Vet. Inst. Pulawy, 56:623-629.

Verheyen K, Price J, Lanyon L & Wood J (2006). Exercise distance and speed affect the risk of fracture in racehorses. Bone, 39:1322-1330.

Yovich JV & McIlwraith CW (1986). Arthroscopic surgery for osteochondral fractures of the proximal phalanx of the metacarpophalangeal and metatarsophalangeal (fetlock) joints in horses. J. Am. Vet. Med. Assoc., 188:273-279.

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Reproductive capacity of male bank voles (*Myodes glareolus* SCHREBER, 1780) - age-dependent changes in functional activity of epididymal sperm

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ABSTRACT. The influence of age on male bank voles' reproductive tract development, epididymal sperm quantity and functional activity was investigated. Experiments were carried out on male bank voles aged 1.5 to 15 months (n=10 each in 8 age groups). The developmental stage of the reproductive tract was assessed by the weight of testes, seminal vesicles and coagulation glands. In each age group the number of epididymal sperm and their functional activity were examined. Epididymal sperm functional activity was assessed by motility, viability, maturity, head morphology and integrity of the sperm tail membrane. Ageing males were heavier than pre-pubertal and mature ones. Male age also affected the testes, seminal vesicles and coagulation gland development. The heaviest accessory sex glands were noted in 3-month-old males and the lightest in pre-pubertal (1.5-month-old) and older (12- and 15-month-old) males. Sperm counts were significantly higher in 3-, 4- and 5-month-old males than in pre-pubertal and old males. Generally, adult males aged 3- and 4- months , produced sperm of better functional activity. In conclusion, the best male reproductive capacity is found in bank voles of 3 to 4 months of age.

KEY WORDS: age, bank vole, spermatozoa, reproductive tract, sperm activity

INTRODUCTION

Interest in the effect of paternal age on physical conditions and reproductive efficiency of progeny has recently increased worldwide. Studies on humans and certain rodents show that among anomalous effects associated with advanced male age are pregnancy losses (SERRE & ROBAIRE, 1998; SLAMA et al., 2005), stillbirth (ASTOLFI et al., 2004) and increased postnatal mortality (ZHU et al., 2008). Mutation frequencies in male genomes also increase with age, which may evoke negative effects on individual health, infertility as well as genetic defects in offspring (for a review, see for example SLOTER et al., 2004). In the Brown Norway rat (*Rattus norvegicus*), ageing leads to considerable damage in the testes and to lower serum testosterone levels due to decreased pituitary hormonal activity, and to decreased sperm production (WANG et al., 1993). In old mice more atrophic testes with degenerated seminiferous tubules have been found (PARKENING et al., 1988).

The efficiency of spermatogenesis is a key to male reproductive success. Spermatozoa concentrations as well as their functional activity are parameters that can be used to assess the potential success of the sperm and, consequently, the reproductive success of males. Functional activity can be assessed by spermatozoa viability, motility, maturity, integrity of the sperm tail membrane and head morphology. The study of age-dependent changes in sperm quality of the black-footed ferret (*Mustela nigripes*) based on only two parameters showed that motile and structurally normal sperm was diminished in older males (WOLF et al., 2000). To our knowledge, there are few published reports assessing the

influence of male age on complex measurements of sperm functional activity. Studies on mice have shown that mating with males of 12 months of age and older significantly reduced fertilization and decreased implantation potential and fetal viability (KATZ-JAFFE et al. 2013). The influence of age has been also demonstrated in studies on the male mosquito, where the optimum male age for successful insemination of females was found to be 4-8 days (SAWADOGO et al. 2013). The aim of the present study was to investigate the effect of age on the reproductive development of bank vole (*Myodes glareolus*) males. The bank vole is presumably the most common small rodent in Europe. This species has become a research object in behavioural, population, and environmental protection studies and it is also used as a classical model for laboratory experiments. Reproductive biology of bank vole is rather well known, including such aspects as time of male sexual maturity (MARCHLEWSKA-KOJ, 2000), factors influencing mating behaviour (MARCHLEWSKA-KOJ et al., 2003) and multiple paternity (RATKIEWICZ & BORKOWSKA, 2000). In the wild, bank voles breed seasonally (CLARKE, 1981). The reproductive season lasts from the beginning of March to the end of October. Males living in a natural environment reach puberty at about two months of age (BUJALSKA, 1973). In a laboratory colony mature spermatozoa were observed for the first time in 1.5-month-old bank vole males (KRUCZEK, 1986). However the optimal male age for reproduction is still not well determined. In the present study we examined the effects of age on the quality and functional activity of epididymal sperm of bank vole males using different parameters.

MATERIALS AND METHODS

Animals

The bank voles used in the experiments came from the Institute of Environmental Sciences, Jagiellonian University in Kraków. The animals are maintained as outbred stock according to the system described by Green (GREEN, 1966). Briefly, each generation consists of at least 22 breeding pairs; the male and female in each mating pair do not have common parents or grandparents. This breeding system ensures the heterogeneity of the colony. It could be assumed that animals from such a colony are comparable to rodents living in a natural population. Bank voles were kept in polyethylene cages (36 x 21 x 17 cm) under a 14 h photoperiod (lights on at 06:00 a.m.) at 20±2° C. Standard pelleted chow and water were available *ad libitum*. Wood shavings were provided as bedding material and changed once a week. The males were housed, at least in 70% of cases, as three per cage from weaning at 19-20 days; only occasionally were there two or four males per cage.

The experiments were carried out on males aged 1.5 (1.5M), 3 (3M), 4 (4M), 5 (5M), 6 (6M), 9 (9M), 12 (12M), and 15 months (15M). There were 10 males in each tested group (n=10), all together 80 males. The males in each age group came from different mating pairs.

Procedures of body and organ weights and of epididymal sperm evaluation

At the appropriate age, the males were sacrificed by cervical dislocation and weighed. The paired testes and seminal vesicles and coagulating glands were dissected out and weighed (the latter two jointly) too. The procedures and methods to count and observe spermatozoa were the same as used for mice (STYRNA & KRZANOWSKA, 1995; STYRNA et al., 2003) as well as for bank voles in our previous paper (KRUCZEK & STYRNA, 2009). The individual counting and analyzing of epididymal sperm were conducted without information on the males' ages.

Preparation of epididymal sperm suspension

After gentle pressing of each cauda epididymis with forceps, allowing sperm to pass to the vasa deferentia, the latter was dissected out and its content gently squeezed directly into 100 µl of M2 medium (Sigma-Aldrich, Germany) containing 2% albumin bovine fraction V,

placed in Petri dishes, and allowed to disperse at room temperature for 2 minutes (STYRNA AND KRZANOWSKA, 1995; KRUCZEK AND STYRNA, 2009).

Epididymal sperm concentration

A 1:20 dilution of epididymal sperm suspension with M2 medium (Sigma) was prepared, and the number of spermatozoa in 100 squares of a hemocytometer was counted under a light microscope at 400x magnification. The average of two epididymal sperm counts was used to estimate sperm concentration. Epididymal sperm functional activity was assessed by spermatozoa motility, sperm tail membrane integrity (hypo-osmotic swelling test), viability, maturity (spermatozoa without cytoplasmic droplet) and sperm head morphology.

Epididymal sperm motility

Spermatozoa motility was assessed in a hemocytometer. A small drop of sperm suspension was transferred to a hemocytometer, covered with coverslip and spermatozoa showing progressive movements were inspected. The number of spermatozoa without a cytoplasmic droplet among 200 counted spermatozoa with progressive movements from each male was reported (SEED et al., 1996).

Epididymal sperm tail membrane integrity – water test

The integrity of the epididymal sperm tail membrane was determined by the hypo-osmotic swelling test (WALCZAK et al. 1994; STYRNA & KRZANOWSKA 1995; KRUCZEK & STYRNA 2009). 20 µl of epididymal sperm suspension (as described in Epididymal sperm concentration) was mixed with 120 µl distilled water on a clean glass slide, then the mixture was gently covered with a coverslip and incubated for 5 min at 37° C before it was examined. The percentage of spermatozoa showing swelling among 200 counted spermatozoa from each male was estimated.

Epididymal sperm viability – eosin-Y test

The test reflects the structural and morphological integrity of the epididymal sperm membrane in human and mouse sperm (WALCZAK et al., 1994; STYRNA & KRZANOWSKA, 1995). To assess epididymal sperm viability, 20 µl of epididymal sperm suspension (as described in *Epididymal sperm concentration*) was mixed with 20 µl of 0.2% eosin Y, incubated for 10 min at 37° C and smeared on a slide. The percentage of spermatozoa with unstained sperm heads (viable spermatozoa) among 200 counted spermatozoa from each male was calculated.

Epididymal spermatozoa without a cytoplasmic droplet

20 µl of sperm suspension (as described in *Epididymal sperm concentration*) was transferred to a slide and gently covered with a coverslip. According to the classification described for mice (STYRNA et al., 2002), three sperm categories can be distinguished: (1) without a droplet, (2) with a droplet on the end of the middle piece (distal droplet), and (3) with a droplet situated more proximally (proximal droplet) (Fig.1). For the present experiments, the percentage of spermatozoa without a cytoplasmic droplet among 200 counted spermatozoa with progressive movements was given for each male.

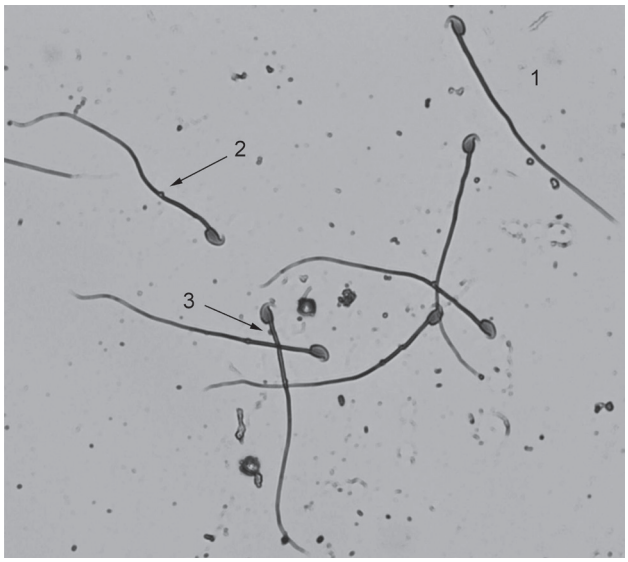

Fig. 1. – Three sperm categories of bank vole males: 1. without a cytoplasmic droplet, 2. with distal cytoplasmic droplet (with a droplet on the end of the middle piece), 3. with proximal cytoplasmic droplet (with a droplet situated more proximally)

Epididymal sperm morphology

For morphological examination a small drop of the epididymal sperm suspension was smeared on a slide, air-dried, fixed in acetic alcohol (absolute alcohol, glacial acetic acid, 3:1), and stained with Papanicolau to assess the proportions of different epididymal sperm head abnormalities. Based on our earlier study (KRUCZEK & STYRNA, 2009) for assessing bank vole epididymal sperm morphology, the following misshapen forms are distinguished : type 1- acrosomal abnormalities; type 2 - abnormalities in the distal part of the head; type 3 - serious abnormalities in the proximal part of sperm heads and type 4 - elliptic head. For statistical treatment, only classes of abnormal spermatozoa with a frequency of more than 0.5% of the total sperm in all tested animals were considered. They formed two classes: class 1 – slightly deformed with a small acrosomal part (type 1); and class 2 – other abnormalities, a group being composed of the remaining sperm defects described above, namely misshapen forms of types 2, 3 and 4.

The experimental procedures for this study were approved by the Regional Committee on Animal Experimentation in Kraków (Protocol No. 26/2007 and 82/2008) acting in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and conformed to the "Guidelines for the use of animals in research" (Animal Behaviour 1991, 41:183-186)

Statistical analysis

The body weight, testes and accessory sex gland weights and epididymal sperm evaluation variables were compared by a one-way analysis of variance (ANOVA), followed by post hoc pair-wise comparisons using Tuckey's tests where the ANOVA gave significant results, as data had normal distributions. For statistical treatment (1) percentages were arcsine transformed (arcsine of the square root of the proportion) (2) the following classes of epididymal sperm morphology were taken as variables: class 1; the sum of classes 2, 3, and 4; and total percentage of abnormal shapes. All results were expressed as means ± S.E.M., and $p < 0.05$ was considered as statistically significant. All procedures were carried out using the Statistica PL ver. 8.0 statistical package.

RESULTS

Body and organ weights

Body weights of males increased with age up to 6 months and more. The 6M, 9M, 12M and 15M males were significantly heavier than 1.5M, 3M and 4M bank voles. Additionally, 3M, 4M and 5M males had significantly bigger body masses than did 1.5M males (Table 1). The relative weights of the testes of 1.5M males were significantly lower compared to other age groups. The biggest value of relative weight of accessory sex organs was noted for 3M males although significant differences were only observed between 3M males and the youngest 1.5M males as well as between 3M and the oldest 12M and 15M males (Table 1).

Epididymal sperm evaluation

Epididymal sperm activity was influenced by male age. Mean sperm concentrations were significantly higher in 3M, 4M and 5M males than in 1.5M, 6M, 9M, 12M and 15M males (Fig. 2). The sperm of 3M and 4M males were characterized by a large proportion of progressive motile spermatozoa and this proportion was significantly larger than in 1.5M, 5M, 6M, 9M, 12M and 15M males (Fig. 3a). Swollen epididymal spermatozoa were significantly less frequent in groups 3M and 4M than in other groups. Moreover, the 5M and 6M bank voles had significantly lower proportions of swollen spermatozoa than did 9M, 12M and 15M males (Fig. 3b).

On the other hand higher proportions of viable epididymal spermatozoa were observed in the

TABLE 1

The age effects on the organometric parameters and proportion of abnormal epididymal sperm in bank vole males (mean ± S.E.M.).

Age groups (months)	Body (g)	Relative weights of (mg/10 g body weight) testes	sem. ves. + coag. gl.
1.5	15.98 ± 1.02^{A,B,C,D,E}	211.65 ± 6.98^{A,B,C,D,E,F,G}	107.17 ± 9.29^{A}
3	24.13 ± 0,94^{A,B,C,D,E}	303.78 ± 11 01^{A}	177.38 ± 13.65^{A,B,C}
4	25.21 ± 0.90^{A,B,C,D,E}	289.46 ± 9.24^{B}	150.09 ± 8.43
5	26.28 ± 1.66^{E,}	307.97 ± 11.57^{C}	145.54 ± 8.70
6	32.08 ± 1.25^{A,}	278.22 ± 7.84^{D}	134.42 ± 4.33
9	31.77 ± 0.92^{B}	321.90 ± 13.57^{E}	134.14 ± 15.12
12	31.75 ± 1.86^{C}	274.30 ± 11.81^{F}	127.33 ± 6.47^{B}
15	31.84 ± 1.33^{D}	296.21 ± 11.49^{G}	113.04 ± 6.64^{C}
$F_{/7.72/}$	19.75	9.99	5.22
p	< 0.01	< 0.01	< 0.01

Means marked by the same letters differ significantly at A–O - $p < 0.01$, a, b - $p < 0.05$.

Abnormal spermatozoa		
total	class 1	class 2
0.16 ± 0.02^{A,D,G,J,M,O}	0.12 ± 0.01^{A,D,G,I,M,O}	0.04 ± 0.01^{A,B,C,a,b}
0.05 ± 0.00^{A,B,C}	0.03 ± 0.00^{A,B,C}	0.02 ± 0.00^{a}
0.03 ± 0.00^{D,E,F}	0.02 ± 0.00^{D,E,F,L}	0.01 ± 0.00^{A}
0.06 ± 0.00^{G,H,I}	0.05 ± 0.00^{G,H,a}	0.01 ± 0.00^{B}
0.06 ± 0.01^{J,K,L}	0.05 ± 0.01^{I,J,K}	0.01 ± 0.00^{C}
0.07 ± 0.01^{M,N}	0.05 ± 0.01^{L,M,N}	0.02 ± 0.00^{b}
0.10 ± 0.01^{B,E,H,K,O}	0.08 ± 0.01^{B,E,J,O,a,b}	0.02 ± 0.00
0.14 ± 0.01^{C,F,I,L,N}	0.12 ± 0.01^{C,F,H,K,N,b}	0.02 ± 0.01
27.46	29.96	4.74
< 0.01	< 0.01	< 0.01

3M and 4M males in comparison with other age groups (Fig.4a). Additionally, the proportion of viable sperm in 3M males was significantly higher than in 5M animals; and 5M males had significantly more viable sperm than did 6M, 9M, 12M and 15M bank voles (Fig. 4a).

Sperm without a cytoplasmic droplet was significantly more frequent in the vasa deferentia of 3M and 4M males than in all other ages, and 15M and 3M males had significantly more mature spermatozoa than did 4M bank voles (Fig. 4b). Additionally, 5M and 6M males had a significantly higher proportion of mature spermatozoa than did 9M, 12M and 15M males; and 1.5M males had significantly more mature spermatozoa than did 12M and 15M males. Finally 9M males had significantly more spermatozoa without a cytoplasmic droplet than did 15M bank voles (Fig. 4b).

There were also significant differences in sperm morphology between age groups of males (Table 1). The mean total proportion of abnormal sperm was significantly lower in 3M, 4M, 5M, and 6M males than in 1.5M, 12M and 15M males. The sperm of 9M animals was characterized by a significantly lower proportion of total abnormal

sperm in comparison with 1.5M and 15M males, and 12M animals had significantly less abnormal sperm than did 1.5M males. Similarly, 3M, 4M, 5M, and 6M males had significantly lower proportions of class 1 abnormalities than did 1.5M, 12M and 15M animals; additionally, the proportion of class 1 abnormalities in 4M bank voles was significantly lower than noted for 9M males. At 9M and 12M there were lower proportions of class 1 abnormalities than at 1.5M and 15M .The sperm of 3M, 4M, 5M, 6M, and 9M males was characterized by significantly lower values of class 2 spermatozoa abnormalities than that of 1.5M males (Table 1).

DISCUSSION

A positive relationship between ageing and increase in body weight has also been documented for Spraque-Dawley rats (see for review WANG et al., 1993) while a negative correlation between body mass and age has been shown for Fisher 344 rats (BASKIN et al., 1979). The high positive dependence between bank vole male body mass and testes weight observed in our experiments are in agreement with earlier observations on bank voles (YLÖNEN et al., 2004). Since the development and secretory activity of the male accessory sex organs are under the direct control of androgens produced by the testes (FRANCA et al., 2006), the growth of these organs in 3-, 4- and 5- months-old bank vole males could be evoked by an increase of testosterone. It has been documented for Brown-Norway rats that ageing in the reproductive system is manifested by lower serum testosterone levels (WANG et al., 1993; HARDY & SCHLEGEL, 2004). A positive role of accessory sex organs on sperm functional activity has been observed in hamsters where secretions protect sperm against oxidative damage influencing sperm viability or motility (CHEN et al., 2002). So for adult bank vole males, it seems to be beneficial to have larger seminal vesicles and coagulating glands.

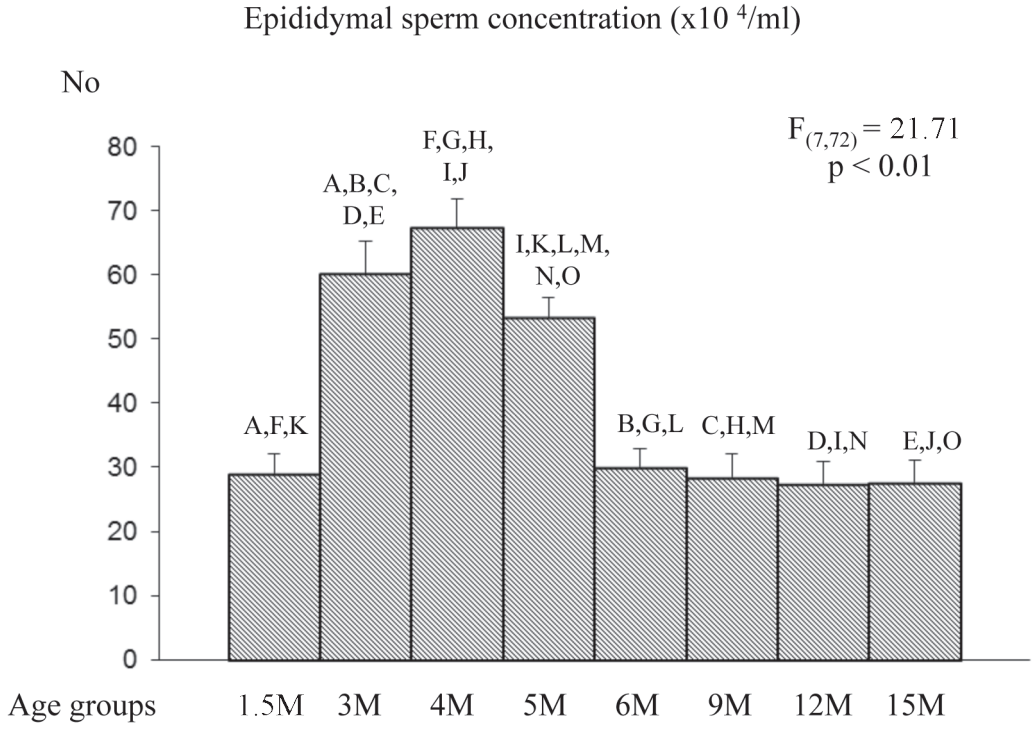

Fig. 2. – Epididymal sperm concentration (x10^4/ml) in 1.5- (1.5M), 3- (3M), 4- (4M), 5- (5M), 6- (6M), 9- (9M), 12- (12M) and 15-month-old (15M) bank vole males (means ± S.E.M.). Number of males in each age group = 10. Means marked by the same letters differ significantly at A-O - $p < 0.01$.

In the present study we also showed that adult bank vole males (3-, 4- and 5-month-old) produced more spermatozoa in comparison to prepubertal and aged males. Considering all parameters assessing epididymal sperm functional activity, i. e. its motility (Fig.3a) , viability (Fig. 4a), and maturity (Fig. 4b), mature males produced sperm of better functional activity. Their sperm

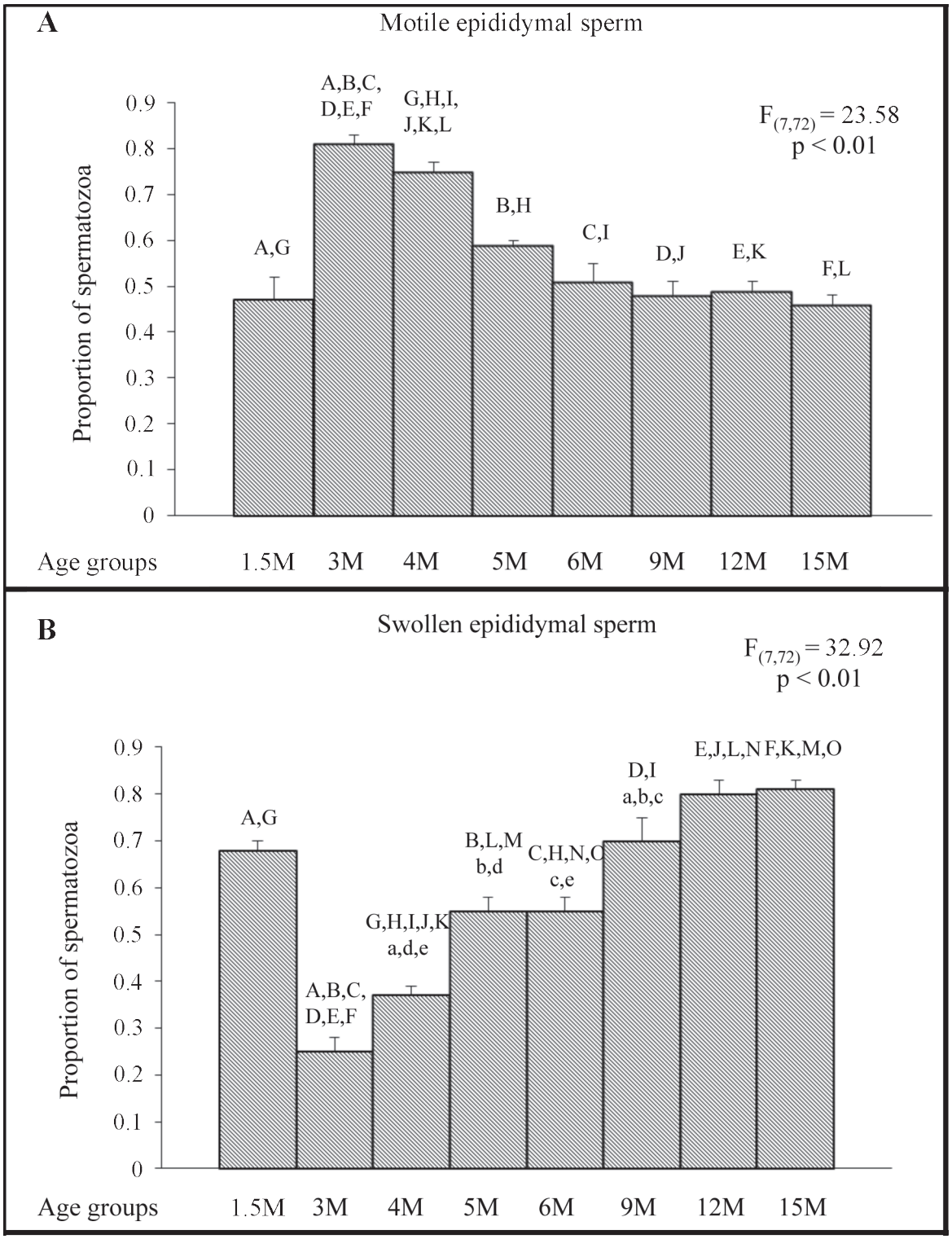

Fig. 3. – Proportions of motile (a) and swollen (b) epididymal sperm in 1.5- (1.5M), 3- (3M), 4- (4M), 5- (5M), 6- (6M), 9- (9M), 12- (12M) and 15-month-old (15M) bank vole males (means ± S.E.M.). Number of males in each age group = 10. Means marked by the same letters differ significantly at A-O - $p < 0.01$, a-e - $p < 0.05$.

also showed fewer morphological abnormalities and higher tail membrane integrity (Fig. 3b). Decline in daily sperm production with ageing has been also noted in Brown-Norway rats with complete cessation of spermatogenesis in very old males (WANG et al., 1993). The opposite

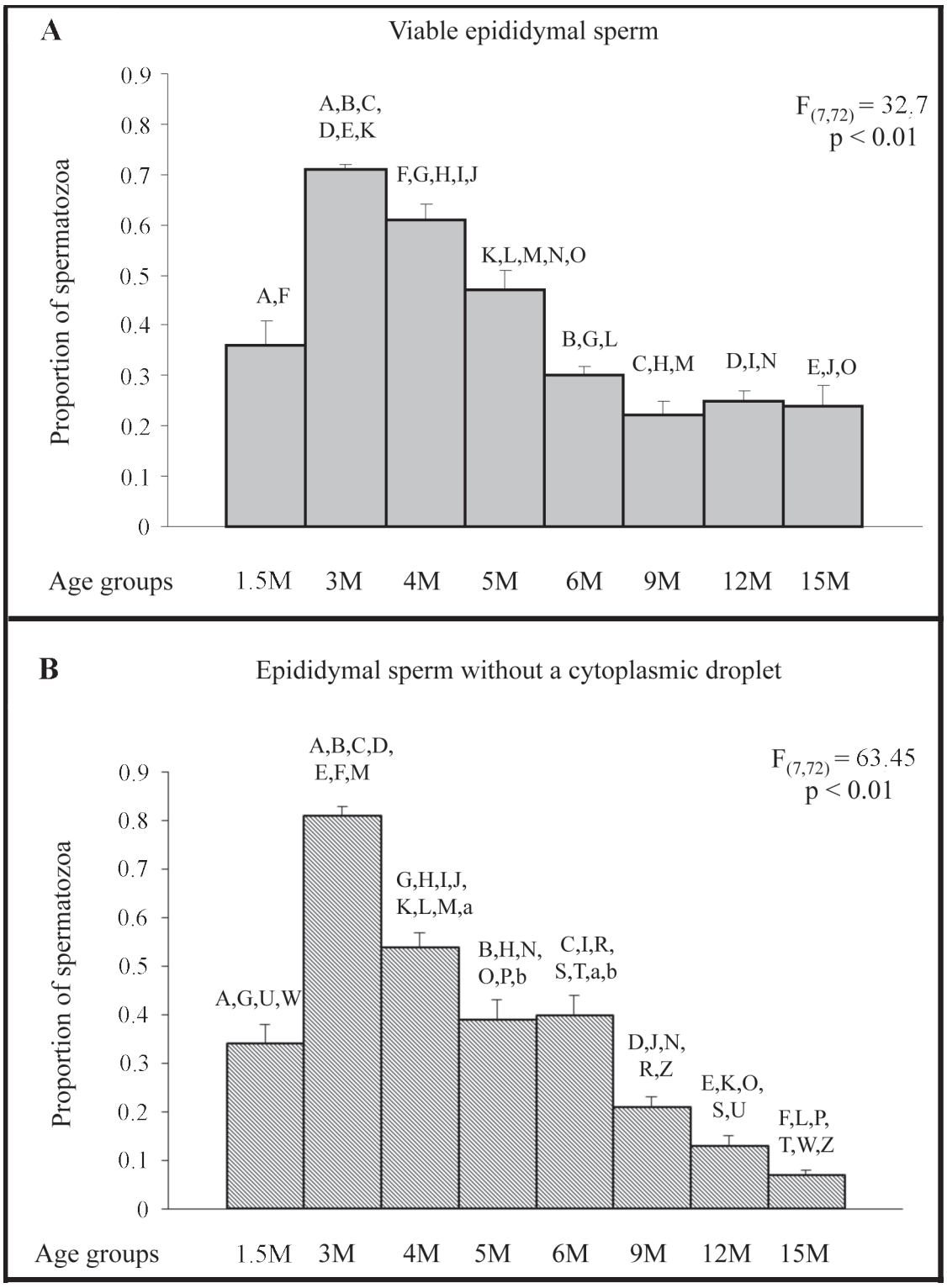

Fig. 4. – Proportions of viable epididymal sperm (a) and epididymal sperm without cytoplasmic droplet (b) in 1.5- (1.5M), 3- (3M), 4- (4M), 5- (5M), 6- (6M), 9- (9M), 12- (12M) and 15-month-old (15M) bank vole males (means ± S.E.M.). Number of males in each age group = 10. Means marked by the same letters differ significantly at A-Z - $p < 0.01$, a-b - $p < 0.05$.

pattern was observed in Spraque-Dowley rats, which did not show progressive decrease in sperm concentration with age (JOHNSON & NEAVES, 1983). Bank voles in captivity live till about 18 months and reproduce till 15 months although their reproductive activity significantly decreased with age (from 6 month) (breeding observation M. Kruczek). In our experiment older bank vole males (to 15 month) do not cease spermatogenesis totally but produce sperm of lower quality.

Progressive movement generated by flagella is necessary for the sperm to reach the ovum in the oviduct and to initiate fertilization (MORTIMER, 1997). In our experiments, the swelling of bank vole spermatozoa, in response to hypo-osmotic solution, was lowest in sperm of adult, 3- and 4-month-old. A strong negative relationship between a high score in the hypo-osmotic test and pregnancy rates has been observed in men (TARTAGNI et al., 2004). On the other hand there was no such correlation in mice (STYRNA & KRZANOWSKA, 1995). As bank voles are more closely related to mice it can be expected that spermatozoa swelling will not have strong influence on pregnancy rate. This aspect of bank vole reproduction has not been investigated before and deserves further study using eg. Sperm Select Penetration Test (STYRNA & KRZANOWSKA 1995).

For several mammalian species correlation between a high proportion of spermatozoa with attached droplets and infertility has been documented (GATTI et al. 2004; COOPER, 2005). Fertilizing potential of spermatozoa depends on their ability to undergo capacitation and acrosome reaction and only viable spermatozoa with intact cell membrane are able to achieve this (YANAGIMACHI, 1981).

As described in Materials and Methods, four categories of misshapen form can be distinguished in bank vole sperm. The most common abnormality is class 1. In this class, acrosomal parts of the head are shorter with very weak staining, which suggests the absence of enzymatic content, which in turn may lead to disorders in acrosomal function and consequent lower male fertility as has been shown in mice (STYRNA et al., 1991, 2002)

Spermatozoa with abnormalities in the distal part of the head, those with serious damage in the proximal part of the head, and spermatozoa with elliptical heads were rarely observed in the bank vole males from each tested age group. Since they did not exceed 0.5% of the total spermatozoa they were pooled together in one class of "other abnormalities". Our results showed that ageing in bank voles produces an increase in the frequency of altered sperm with acrosomal damage as well as in the number of other abnormal forms of spermatozoa. In hamsters, the number of abnormal spermatozoa dramatically increased in advanced-age males from 14-19% in adult to 39-50% in old males, but this increase was due more to an increase in the frequency of altered sperm than through an increase of the other misshapen forms (CALVO et al., 1997). How the misshapen head morphology influences the fertility rate is still under discussion but it is generally accepted that specific head abnormalities correlate with male infertility (for reviews see WEISSENBERG et al., 1987; PESCH & BERGMANN, 2006).

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REFERENCES

ASTOLFI P, DE PASQUALE A & ZONTA LA (2004). Late paternity and stillbirth risk. Human Reproductive, 19:2497-2501.

BASKIN SI, ROBERTS J & KENDRICK ZV (1979). Effect of age on body weight, heart rate and blood pressure in pair-caged male Fisher 344 rats. Age, 2:47-50.

Bujalska G (1973). The role of spacing behaviour among females in the regulation of reproduction in the bank vole. Journal of Reproduction and fertility. Supplement, 19:465-474.

Calvo A, Martinez E, Pastor L, Vazquez J & Roca J (1997). Classification and quantification of abnormal sperm along the epididymal tract. Comparison between adult and aged hamsters. Reproduction Nutrition Development, 37:661-673.

Chen H, Hardy MP & Zirkin BR (2002). Age-related decreases in Leydig cell testosterone production are not restored by exposure to LH in vitro. Endocrinology, 143:1637-1642.

Clarke JR (1981). Physiological problems of seasonal breeding in eutherian mammals. In: Finn CA (ed), Oxford Reviews of Reproductive Biology 3, Clarendon Press, Oxford:244-312.

Cooper TG (2005). Cytoplasmic droplets: the good, the bad or just confusing. Human Reproductive, 20:9-11.

Franca LR, Suescun MO, Miranda JR, Giovambattista A, Perello M, Spinedi E & Calandra RS (2006). Testis structure and function in a nongenetic hyperadipose rat model at prepubertal and adult ages. Endocrinology, 147:1556-1563.

Gatti JL, Castella S, Dacheux F, Ecroyd H, Metayer S, Thimon V & Dacheux JL (2004). Post-testicular sperm environment and fertility. Animal Reproduction Sciences, 82-83:321-339.

Green EL (1966). Breeding systems. In: Green EL (ed), Biology of the Laboratory Mouse. McGraw-Hill Book Company, New York:13-14.

Hardy MP & Schlegel PN (2004). Testosterone production in the aging male: where does the slowdown occur? Endocrinology, 145:4439-4440.

Johnson L & Neaves WL (1983). Enhanced daily sperm production in the remaining testis of aged rats following hemicastration. Journal of Andrology, 4:162-166.

Katz-Jaffe MG, Parks J, McCallie B & Schoolcraft WB (2013). Aging sperm negatively impacts in vivo and in vitro reproduction: a longitudinal murine study. Fertility and Sterility, 100:262-268.e2

Kruczek M (1986). Seasonal effects on sexual maturation of male bank voles (*Clethrionomys glareolus)*. Journal of Reproduction & Fertility, 76:83-89.

Kruczek M & Styrna J (2009). Semen quantity and quality correlate with bank vole males' social status. Behavioural Processes, 82:279-285.

Marchlewska-Koj A (2000). Olfactory and ultrasonic communication in bank voles. Polish Journal of Ecology, 48:11-20.

Marchlewska-Koj A, Kruczek M & Olejniczak P (2003). Mating behaviour of bank voles (Clethrionomys glareolus) modified by hormonal and social factors. Mammalian Biology , 68:144-152.

Mortimer ST (1997). A critical review of the physiological importance and analysis of sperm movement in mammals. Human Reproduction Update, 3:403-439.

Parkening TA, Collins TJ & Au WW (1988). Paternal age and its effects on reproduction in C57BL/6NNia mice. Journals of Gerontology, 43:79-84.

Pesch S & Bergmann M (2006). Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. Micron, 37:597-612.

Ratkiewicz M & Borkowska A (2000). Multiple paternity in the bank vole (*Clethrionomys glareolus*): field and experimental data. Mammalian Biology, 65:6-14.

Sawadogo SP, Diabaté A, Toé HK, Sanon A, Lefevre T, Baldet T, Gilles J, Simard F, Gibson G, Sinkins S, Dabiré RK (2013). Effects of age and size on Anopheles gambiae s.s. male mosquito mating success. Journal of Medical Entomology, 50:285-293.

Seed J, Chapin RE, Clegg ED, Dostal LA, Foote RH, Hurtt ME, Klinefelter GR, Makris SL, Perreault SD, Schrader S, Seyler D, Sprando R, Treinen KA, Veeramachaneni DN & Wise LD (1996). Methods for assessing sperm motility,morphology, and counts in the rat, rabbit, and dog: a consensus report. Reproductive Toxicology, 10:237-244.

Serre V & Robaire B (1998). Paternal age affects fertility and progeny outcome in the Brown Norway rat. Fertility & Sterility, 70:625-631.

Slama R, Bouyer J, Windham G, Fenster L, Werwatz A & Swan SH (2005). Influence of paternal age on the risk of spontaneous abortion. American Journal of Epidemiology, 161:816-823.

Sloter E, Nath J, Eskenazi B & Wyrobek AJ (2004). Effects of male age on the frequencies

of germinal and heritable chromosomal abnormalities in humans and rodents. Fertility & Sterility, 81:925-942.

STYRNA J, BILIŃSKA B & KRZANOWSKA H (2002). The effect of a partial Y chromosome deletion in B10.BR-Ydel mice on testis morphology, sperm quality and efficiency of fertilization. Reproduction, Fertility & Development, 14:101-108.

STYRNA J, IMAI, HT & MORIWAKI K (1991). An increased level of sperm abnormalities in mice with a partial deletion of the Y chromosome. Genetics Research, 57:195-199.

STYRNA J, KILARSKI W & KRZANOWSKA H (2003). Influence of the CBA genetic background on sperm morphology and fertilization efficiency in mice with a partial Y chromosome deletion. Reproduction, 126:579-588.

STYRNA J & KRZANOWSKA H (1995). Sperm select penetration test reveals differences in sperm quality in strains with different Y chromosome genotype in mice. Archives of Andrology, 35:111-118.

TARTAGNI M, CICINELLI E, SCHONAUER MM, CAUSIO F, PETRUZZELLI F & LOVERRO G (2004). Males with subnormal hypo-osmotic swelling test scores have lower pregnancy rates than those with normal scores when ovulation and timed intercourse is used as a treatment for mild problems with sperm count, motility, or morphology. Journal of Andrology, 25:781-783.

WALCZAK R STRUMILLO E & KULA K (1994). Eosin and water tests and results of conventional semen analysis. Ginekologia Polska, 65:99-102.

WANG C, LEUNG A & SINHA-HIKIM AP (1993). Reproductive aging in the male brown-Norway rat: a model for the human. Endocrinology, 133:2773-2781.

WEISSENBERG R, BELLA R & LUNENFELD B (1987). The fertilization capacity of golden hamster epididymal spermatozoa in relation to age, number, motility and morphology. Andrologia, 19:47-53.

WOLF KN, WILDT DE, VARGAS A, MARINARI PE, KREEGER JS, OTTINGER MA & HOWARD JG (2000). Age-dependent changes in sperm production, semen quality, and testicular volume in the black-footed ferret (*Mustela nigripes*). Biology of Reproduction, 63:179-187.

YANAGIMACHI R (1981). Mechanisms of fertilization in mammals. In: Mastroianni L&, Biggers JD (eds). Fertilization and embryonic development in vitro. Plenum Press. New York:81-182.

YLÖNEN H, HORNE T.J & LUUKKONEN M (2004). Effect of birth and weaning mass on growth, survival and reproduction in the bank vole. Evolutionary Ecology Research, 6:1-10.

ZHU JL, VESTERGAARD M, MADSEN KM & OLSEN J (2008). Paternal age and mortality in children. European Journal of Epidemiology, 23:43-447.

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SHORT NOTES

Successful reproduction of Hen Harrier *Circus cyaneus* in intensive arable farmland (central-east Belgium)

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KEY WORDS: Agrobiodiversity, Birds Directive, Colonization, Farmland birds, Monitoring, Raptor research, Species protection program

In many European countries the number of Hen Harrier *Circus cyaneus* breeding pairs has declined during the second half of the twentieth century (1, 2, 3). Because of the danger for regional extinction, the European Union (EU) protected the species under the Birds Directive in 1979 and set up the Natura 2000 conservation program (4).

The total European breeding population has been estimated at 32,000-59,000 pairs (2). In France, which is Western Europe's stronghold for the species with 7,800-11,200 pairs, the species seems on the rise with many breeding pairs shifting from natural to more cultivated areas during the past decades (5, 6). As for The Netherlands, recently a few pairs started to breed in Groningen's farmland, probably facilitated by the presence of fauna strips (Stichting Werkgroep Grauwe Kiekendief, unpublished data). In Belgium, Hen Harrier was and still is a very rare breeding bird. Most known breeding occurrences of the last 40 years were located in natural habitats (open areas, such as bogs and fens) in the southern part of the country (Wallonia) (3, 7). From 2002 onwards, breeding attempts have taken place each year in Wallonia, some of them in agricultural land, with three confirmed and two probable breeding pairs in 2011 (Vincent Leirens, unpublished data). In Flanders (northern Belgium), the species has not bred for the last 40 years (8, 9). Only in the last decade has suspected breeding of Hen Harrier in Flanders reoccurred.

In this contribution we document the first breeding occurrences of Hen Harrier in a previously unoccupied agricultural region in central-east Belgium (Outgaarden, N 50°46' E 04°55'). By following breeding efforts for six consecutive years (2006-2011), we tried to get insights into the ecological requirements of the species in this intensive arable farmland. Information on the breeding and foraging habitat was gathered, allowing optimization of conservation measures for better protection of the species, hopefully resulting in further colonization of the region.

Information on the number of males and females, the number and location of nests, and the number of fledged young was collected by a team of two to five volunteer ornithologists in each study year. Breeding places were located on an orthophoto in a GIS environment, and complemented with information on yearly agricultural crops for each parcel and other present land use (forest, urban & infrastructure) (Agentschap voor Landbouw en Visserij, unpublished data; Direction Générale de l'Agriculture, des Ressources Naturelles

TABLE 1

Summarizing table presenting, for each survey year, the number of involved Hen Harrier.

Year	Females	Nests		Fledged young
		Failed	Successful	
2006	1	0	1	1
2007	1	0	1	3
2008	2	2	1	2
2009	2	2	0	0
2010	1	0	1	5
2011	1 or 2	1 or 2	0	0
Total	8 or 9	5 or 6	4	11

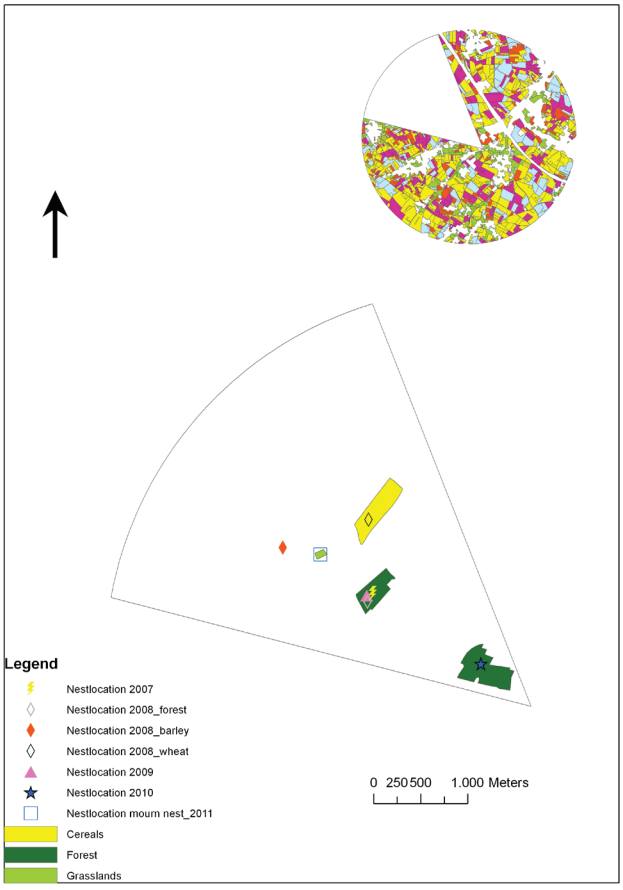

Fig. 1. – Location of all Hen Harrier nests (2006-2011). The map is drawn with a radius of 4,000 m around the 2010 nest. The outset graph depicts the larger setting of the nests (i.e. location of the 2010 nest with a radius of 4,000 m; see Fig. 3 right).

ET DE L'ENVIRONNEMENT, unpublished data). For each nest of every year, circles with a radius of 350 m (the area around the nest that is normally defended against predators), 2,000 m (suitable foraging habitat close to the nest and where most hunting takes place), and 4,000 m (the general habitat up to where the male occasionally hunts) around the nests were drawn, and the surface area of land uses and crops determined (see also 10, 11). The agricultural data were merged into seven groups, being cereals, corn, permanent and temporary grasslands, potatoes, sugar beets and other crops (mainly consisting of many types of vegetables and fruits).

Over the six-year period, 10 breeding cases were initiated by a yearly average of 1.5 females (Table 1). Four nests were successful and a total of 11 young fledged. In 2006, one young bird fledged, while the next year (2007) three young were raised in a small forest patch of around 9 ha (Fig. 1). In 2008, a breeding case was initiated in the same forest, but failed. A replacement nest was made in a nearby barley field, with two young successfully growing up. In 2009, no nests were successful, while in 2010 five young fledged from a nest located in an adjacent forest patch of around 16 ha. In 2011 no nests were successful, with a so-called 'mourn nest' (quickly constructed and soon abandoned) being built by the male in a nearby meadow. In general, the fledged juveniles left the area during August, in the same period when the first migrant Hen Harriers were seen.

In most of the years breeding took place in isolated deciduous forest patches (Atlantic acidophilous beech forests; habitat type 9120 according to the Habitats Directive). In these forest patches, nests were made in impenetrable and high creeping Bramble thickets *Rubus fruticosus* amidst clearings (Fig. 2). The nests were surrounded by an average of 70 % annual crops in a 4,000 m radius around the nests. In both the 2,000 and 4,000 radiuses, only very small differences were observed over the years for the percentage of occurring land cover classes (cereals: 38 % SE 2, potatoes and sugar beets:

Fig. 2. – Overview picture of the 2010 Hen Harrier nest in a small deciduous forest patch. The nest is located in Bramble thickets amidst a clearing, with in front some hiding chicks.

17 % SE 1, urban: 14 % SE 1, other crops: 12 % SE 1, corn: 8 % SE 1, grasslands: 6 % SE 0, forest: 5 % SE 1; Fig. 3).

The colonization of intensive arable farmland in central-east Belgium by Hen Harrier seems at first sight quite remarkable. Hen Harrier, in Western European countries typically a bird of natural open areas, now cautiously takes a step to adjust and settle in some large-scale agricultural regions. These farmland regions suffered from land use intensification, which generally resulted in a decreased availability of prey (12). To counteract the drop of farmland biodiversity, occasional agrobiodiversity measures have been established throughout Europe, but their effectiveness remains controversial (13, 14). However, for the specific region of Outgaarden there are indications that the former EU agricultural policy of set-aside land, in combination with well-established agrobiodiversity measures, may have resulted locally in an increase of prey and target farmland biodiversity (15). Additionally, individuals from nearby northern France populations seeking new territories (the nearest French breeding populations are located at less than 100 km from Outgaarden) (6), may explain the recent

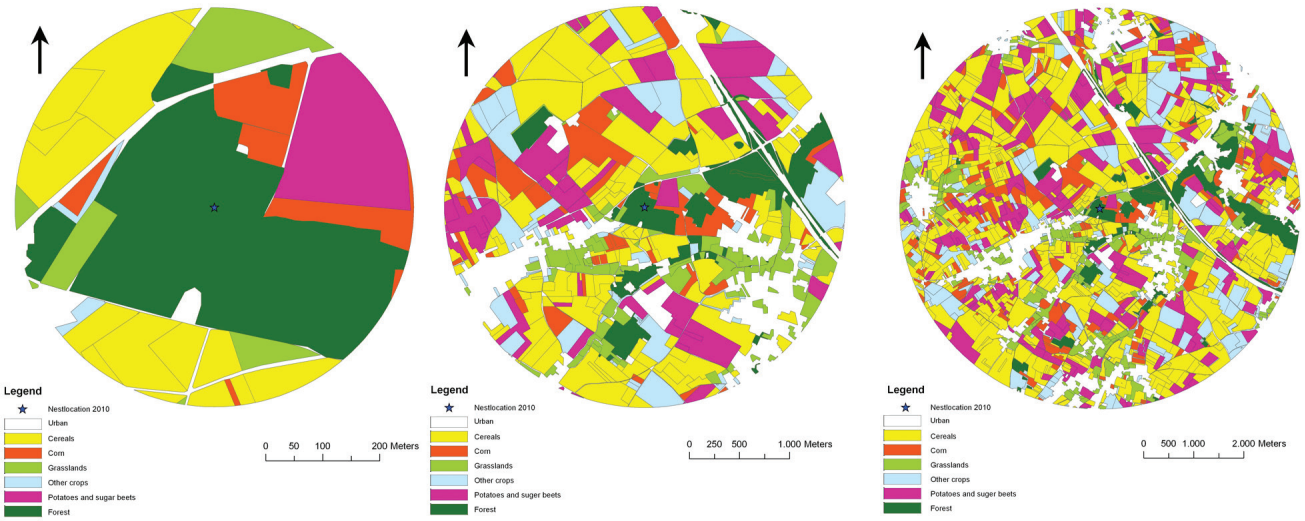

Fig. 3. – Location of the Hen Harrier nest of 2010 with a radius of 350 m around the nest (left), 2,000 m (middle), and 4,000 m (right).

colonization. Nevertheless, individuals in the region may originate from further afield than just adjacent France. For instance, a female ringed as a chick in a salt marsh on a German Wadden Sea Island in 2009, was seen near her successful nest in Wallonia in summer 2011, about 400 km from her birthplace (Vincent Leirens, unpublished data).

Abroad, Hen Harriers present in an intensively cultivated area in the Champagne-Ardenne (France) breed now and then in young woodlands, with the majority using cereal fields (5). On the Dutch Wadden Sea islands, males prefer dune thickets and open dunes for hunting. There, they have a strong preference for non-grazed areas (16). In Groningen (The Netherlands), Hen Harriers recently colonized farmland, where the breeding habitat consists of cereal fields and alfalfa, containing many fauna strips throughout the area (STICHTING WERKGROEP GRAUWE KIEKENDIEF, unpublished data). On the Orkney Islands (UK), most Hen Harriers breed in Heather *Calluna vulgaris* on moorland (17), but males seem to prefer unmanaged rough grass for hunting (18). By contrast, in Ireland, 75 % of the Hen Harrier breeding population chose second rotation plantation forest as nesting habitat (19). These breeding locations contrast considerably with the situation in Outgaarden, as in most years Hen Harriers chose to breed in small forest patches instead of nesting in the abundant cereal fields. This choice is probably due to their early breeding season. During the mating and nesting period (April-early May), the regular (annual) crops (such as winter wheat and barley) provide insufficient cover. In the open forests however, they find the cover they need. Later in the season, when replacement clutches are laid or beta-females start to breed, nests are made in crops that provide enough cover by then.

To counteract the negative tendency in farmland bird abundances, policymakers initiated agrobiodiversity measures in the wider area of Hesbaye (20). Agro-environment schemes started six years ago, with the first measures taken in 2007, and now reaching a few hundred ha in the wider region. These agrobiodiversity measures may additionally have facilitated the breeding of Hen Harrier, after the agricultural set-aside land policy ceased a few years ago. Agrobiodiversity measures consist of broad strips, where a favourable mixture of grasses (leading to improved structure and food supply) is sown, and that are managed through a phased extensive mowing regime. Another important factor in the reproductive success of Hen Harriers may be the presence of farmland nature reserves. In these reserves, untreated spring wheat is sown, which is not mown or sprayed for a year. This may stimulate a high density of rodents (mainly voles), and makes these reserves an ideal hunting area for harriers throughout the year. More specifically, optimal food conditions in the pre-lay period (April) may be crucial for successful nesting. During this period, the male is bound to find enough food so that the female can be piloted in good breeding condition, and clutch formation is strongly influenced by the abundance of food during this period (18). By adjusting the mowing time of grasslands next to extensive management of arable fields, prey may be concentrated on accessible locations when the need for food is at its peak. Whether breeding is successful, however, depends on more, often non-controllable factors, such as age and previous breeding experience of the birds, weather, disturbance, predation, and persecution (21, 22, 23). Analysis of the ecological requirements of the species, although based on a single, but profound survey near Outgaarden, suggests that many regions in Belgium could support breeding Hen Harriers. Detecting the most promising areas in Belgium, and installing suitable agro-environment schemes would probably improve the chances on colonization and conservation of the species on a regional scale.

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REFERENCES

1. Amar A & Redpath S (2002). Determining the cause of the Hen Harrier decline on the Orkney Islands: an experimental test of two hypotheses. Animal Conservation, 5: 21-28.
2. Birdlife International (2004). Birds in Europe: population estimates, trends and conservation status. Birdlife International, Cambridge.
3. Jacob J-P (2010). Busard Saint-Martin, *Circus cyaneus*. In: Jacob J-P, Dehem C, Burnel A, Dambiermont J-L, Fasol M, Kinet T, van der Elst D & Paquet J-Y, Atlas des oiseaux nicheurs de Wallonie 2001-2007. Aves et Région wallonne, Gembloux: 180-181.
4. Louette G, Adriaens D, Adriaens P, Anselin A, Devos K, Sannen K, Van Landuyt W, Paelinckx D & Hoffmann M (2011). Bridging the gap between the Natura 2000 regional conservation status and local conservation objectives. Journal for Nature Conservation, 19: 224-235.
5. Millon A, Bourrioux J-L, Riols C & Bretagnolle V (2002). Comparative breeding biology of Hen Harrier and Montagu's Harrier: an 8-year study in north-eastern France. Ibis, 144: 94-105.
6. Millon A & Bretagnolle V (2004). Busard Saint-Martin, *Circus cyaneus*. In: Thiollay J-M & Bretagnolle V, Rapaces nicheurs de France. Distribution, effectifs et conservation. Delachaux et Niestlé, Paris: 66-69.
7. Hanus B & Dewolf P (1997). Chronique d'une nidification réussie de Busard Saint-Martin (*Circus cyaneus*) en Wallonie. Aves, 34: 39-45.
8. Vlavico 1989. Vogels in Vlaanderen: voorkomen en verspreiding. VLAVICO, Bornem: 121-122.
9. Vermeersch G, Anselin A, Devos K, Herremans M, Stevens J, Gabriëls J & Van der Krieken B (2004). Atlas van de Vlaamse broedvogels: 2000-2002. Mededeling van het Instituut voor Natuurbehoud 23, Brussel.
10. Cramp S (1980). Handbook of the birds of Europe, the middle east, and north Africa: the birds of the Western Palearctic. Vol. 2: Hawks-Bustards. Oxford University, Oxford.
11. Arroyo B, Leckie F & Redpath S (2006). Habitat use and range management on priority areas for Hen Harriers: report to Scottish Natural Heritage. CEH, Banchory.
12. Butet A, Michel N, Rantier Y, Comor V, Hubert-Moy L, Nabucet J & Delettre Y (2010). Responses of Common Buzzard (*Buteo buteo*) and Eurasian Kestrel (*Falco tinnunculus*) to land use changes in agricultural landscapes of Western France. Agriculture, Ecosystems and Environment, 138: 152-159.
13. Kleijn D & Sutherland WJ (2003). How effective are European agri-environment schemes in conserving and promoting biodiversity? Journal of Applied Ecology, 40: 947-969.
14. Kleijn D, Rundlöf M, Scheper J, Smith HG & Tscharntke T (2011). Does conservation on farmland contribute to halting the biodiversity decline? Trends in Ecology and Evolution, 26: 474-481.
15. Guelinckx R et al. (2012). Graan voor gorzen in Natuurpunt Velpe-Mene – Akkerreservaten (Internet address: http://www.velpe-mene.be/nbakkerreservaten.htm).
16. Klaassen O, Dijksen L, de Boer P, Willems F, Foppen R & Oosterbeek K (2006). Broedsucces, voedselecologie en dispersie van de Blauwe Kiekendief op de Waddeneilanden in 2004-2006. SOVON-onderzoeksrapport 2006/15, SOVON Vogelonderzoek Nederland, Beek-Ubbergen.
17. Redpath S, Madders M, Donnelly E, Anderson B, Thirgood S, Martin A & McLeod D (1998). Nest site selection by Hen Harriers in Scotland. Bird Study, 45: 51-61.
18. Amar A & Redpath S (2005). Habitat use by Hen Harriers *Circus cyaneus* on Orkney: implications of land use change for this declining population. Ibis, 147: 37-47.
19. Wilson MW, Irwin S, Norriss DW, Newton SF, Collins K, Kelly TC & O'Halloran J (2009). The importance of pre-thicket conifer plantations for nesting Hen Harriers *Circus cyaneus* in Ireland. Ibis, 151: 332-343.

20. Strubbe D, Verschelde P, Hens M, Wils C, Bauwens D, Dermout M & De Bruyn L (2010). Impact van PDPO II maatregelen op de biodiversiteit. Instituut voor Natuur- en Bosonderzoek, Brussel.
21. Green RE & Etheridge B (1999). Breeding success of the Hen Harrier *Circus cyaneus* in relation to the distribution of grouse moors and the Red Fox *Vulpes vulpes*. Journal of Applied Ecology, 36: 472-483.
22. Parish DMB, Thompson PS & Coulson JC (2001). Effects of age, cohort and individual on breeding performance in the Lapwing *Vanellus vanellus*. Ibis, 143: 288-295.
23. Amar A, Davies J, Meek E, Williams J, Knight A & Redpath S (2011). Long-term impact of changes in sheep *Ovis aries* densities on the breeding output of the Hen Harrier *Circus cyaneus*. Journal of Applied Ecology, 48: 220-227.

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The round goby *Neogobius melanostomus* (PALLAS, 1814) (Perciformes: Gobiidae), an invasive species in the Albert Canal (Belgium)

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Over the last two decades several exotic invertebrate species of different origins have appeared (or reappeared) in the Albert Canal: crustaceans such as the Chinese mitten crab - *Eriocheir sinensis* (MILNE-EDWARDS, 1854) -, the spiny-cheek crayfish - *Orconectes limosus* (RAFINESQUE, 1817) -, the Iberian dwarf shrimp - *Atyaephyra desmaresti* (MILLET, 1831) -, the killer shrimp - *Dikerogammarus villosus* (SOWINSKY, 1874) - and bivalves such as the Asiatic clam - *Corbicula fluminea* (MÜLLER, 1774) -, and the quagga mussel - *Dreissena rostriformis bugensis* (ANDRUSOV, 1897). Some of those species became invasive. Recently, two exotic fish species have also appeared in the canal: the round goby *Neogobius melanostomus* (PALLAS, 1814) since 2010 (1) and the bighead goby *Neogobius kessleri* (GÜNTHER, 1861), the latter first recorded in Belgium on June 13th, 2012 (2).

The round goby is native to the Ponto-Caspian area, where it is a euryhaline benthic species of some economic importance (3). *Neogobius melanostomus* was transported with ballast water of cargo ships to the region of the Great Lakes in North America and to different countries in Central, East, North and West Europe (4, 5, 6). In the Netherlands the round goby was found for the first time in 2004 in the River Lek (7). It was reported for the first time in Belgium on April 8th, 2010 from the River Scheldt near the Liefkenshoektunnel (1). The round goby probably reached the River Scheldt in the ballast water of cargo ships. The species is also known from different places in the Albert Canal, which is connected to the river Scheldt at Antwerp. Since the canal is far too deep for electric fishery

Fig. 1. – Male specimen of *Neogobius melanostomus* from the Albert Canal at Grobbendonk (TL = 137 mm), exhibiting the characteristic black spot on the first dorsal fin. Specimen not preserved.

TABLE 1

Morphometric data of *Neogobius melanostomus* of the Albert Canal at Merksem and Grobbendonk. Abbreviations: TL = total length; SL = standard length; mm = millimeter; OL = otolith length; OH = otolith height. All measurements are in millimeters.

	TL	SL	Sex	Weight in grams	Locality	OL	OH
N. melanostomus	160	137	female	57	Merksem	4,65	2,94
	157	134	male	53	Grobbendonk	4,94	3,24
	146	122	male	49	Grobbendonk	4,59	3,35
	143	123	male	47	Grobbendonk	4,65	2,88
	140	120	male	38	Grobbendonk	4,06	2,71
	138	118	male	40	Grobbendonk	4,53	2,76
	138	116	male	32	Grobbendonk	4,18	2,88
	137	115	male	38	Grobbendonk	4,24	2,76
	132	110	male	31	Grobbendonk	4,41	2,82
	131	112	male	32	Grobbendonk	3,94	2,76
	120	102	male	23	Grobbendonk	4,06	2,59
	120	102	female	21	Grobbendonk	3,76	2,53
	110	95	female	18	Grobbendonk	3,18	2,29
	110	90	male	16	Grobbendonk	3,76	2,82
	105	91	female	12	Grobbendonk	3,56	2,65
	105	85	male	12	Merksem	3,41	2,59
	85	70	male	6	Grobbendonk	2,65	1,94
	83	67	male	6	Grobbendonk	2,41	2,06
N. kessleri	110	92	unknown	unknown	Danube, Vienna	3,94	2,76
	91	77	unknown	unknown	Danube, Vienna	3,12	1,95

and since, according to INBO (the Belgian Research Institute for Nature and Forests), round goby catches in fyke nets are very poor, most data come from anglers (VERREYCKEN, 2012, personal information); indeed at Grobbendonk in locations where round goby catches with fishing-rods were plentiful, the catches with fyke nets placed by INBO were nil.

Fig. 2. – Map showing the Albert Canal in Belgium and sampling sites (Merksem and Grobbendonk).

N. melanostomus has a fairly round head, and rather large eyes, which are slightly protruding from the top of the head. The pelvic fins are fused and form a single pelvic suction disc. A good diagnostic characteristic is the posterior dark spot of the first dorsal fin (Fig. 1), which is absent in the other species of Ponto-Caspian gobies in West Europe. There are 49-55 scales on the lateral line (1). Adult round gobies are mottled with olive green, brown and black irregular spots, whereas juvenile specimens are grey in colour.

Males and females of the round goby can be distinguished by their urogenital papilla: it is white to grey, long and pointed in males and brown, short and blunt tipped in females (4). During the spawning season they exhibit sexual

dimorphism; the males turn black and their cheeks become swollen. They are territorial and display male parental care (guarding the nests with eggs and hatchlings). Spawning can take place in fresh water as well as in salt water (8). The females can spawn up to six times during the spawning season. This results in a fairly high reproduction rate and enhances rapid distribution of this invasive species.

In August 2012 we found two new locations colonized by the round goby in the Albert Canal (Belgium): at Grobbendonk (51° 10' 47.2" N – 04° 45' 17.4" E) and at Merksem (51° 14' 06.6" N – 04° 26' 36.4" E) (Fig. 2). At both sites the species was already fairly abundant: 16 specimens at Merksem and 52 specimens at Grobbendonk were caught on a bank section of 5 m each. Of these, five are preserved at the RBINS (IG 32254 reg. 24951-24954 (Grobbendonk) and IG 32254 reg. 24955 (Merksem)), and 18 were used to collect biometric data (Table 1) and to extract otoliths (see below). The length of the measured specimens varied between 85 mm and 160 mm, whereas the maximum reported length is 220 mm (8).

The distribution of the round goby in the Albert Canal seems to be discontinuous. Apart from the sampling areas, where they are abundant, there are large parts in the canal where they are absent: *e.g.* at Grobbendonk not one single round goby was caught outside the section mentioned above in the first year. The fact that both new locations lay near regularly used mooring places of barges, pleads for the distribution of the round goby by disposal of ballast water by those barges in the Albert Canal. The construction of underwater bank protection with stone debris in that canal (2) probably also facilitates the sustainability of the round goby, because that kind of bank protection forms a suitable habitat for *Dreissena* sp., a regular prey for round gobies. Follow up in future years will determine whether the round goby is able to fill the gaps in its distribution by natural dispersal. In the second year, wider dispersal has already been confirmed by catches at five new locations at Grobbendonk of which only one location was a mooring place.

The round goby has recently been reported from two locations in the River Meuse in Belgium: one near the Dutch border and one near Liège (Verreycken 2013, personal information). This means, that the species is now present in both the rivers Scheldt and Meuse in Belgium, which are connected by the Albert Canal. It had already been reported from the Dutch part of the river Meuse earlier (RAVON, http://www.ravon.nl/Soorten/Vissen).

Round gobies can feed all day long (4), but at Grobbendonk and at Merksem round goby catches with fishing-rods decreased almost to nil after dark. In freshwater the diet of the round goby consists of worms, small crustaceans, insect larvae, molluscs, fish eggs, fish larvae and small fish (8, 9, 10, 11). That diet puts them in competition for food with the native bullhead species - *Cottus perifretum* Freyhof, Kottelat & Nolte, 2005 and *Cottus rhenanus* Freyhof, Kottelat & Nolte, 2005 - resulting in a possible decline of the latter species (11). In the Albert Canal juvenile quagga mussels and juvenile zebra mussels - *Dreissena polymorpha* (Pallas, 1771) - form important prey of the round goby: the smallest shells are swallowed whole, whereas the larger ones are crushed with the laryngeal teeth. In fact, each time that we put some freshly caught round gobies in a clean aquarium, we found a few hours later some small bivalve shells and shell debris of larger specimens of dreissenids on the bottom. So far three juvenile doublets of the Asiatic clam have also been found. Since some pieces of that shell debris are far too large - larger than 1 cm - and too sharp to pass through the intestines or the anus, we presume that at least the large pieces were vomited out by the round gobies after the digestion of the soft parts. In nature, juveniles of those two exotic invasive species of *Dreissena* thus constitute an important part of the food of round gobies, which is probably the only positive aspect of the presence of the latter species in West European waters.

TABLE 2

List of distinguishing features in the otoliths between *Neogobius kessleri* and *Neogobius melanostomus*.

Feature	*N. melanostomus*	*N. kessleri*
(1) dorsal rim	crenelated only in large specimens, more convex with deep notch	crenelated, hardly convex, no notch
(2) postdorsal process	well below highest point of the sagitta	at the same level as the highest point of the sagitta
(3) ventral rim	concave posteriorly	straight
(4) anterior rim	high	not high
(5) sulcus (ostium)	ostium: crista superior convex, crista inferior concave	crista superior and inferior not convex

Potential predators of the round goby in the Albert Canal are pike - *Esox lucius* LINNAEUS, 1758 -, perch - *Perca fluviatilis* LINNAEUS, 1758 -, zander - *Sander lucioperca* (LINNAEUS, 1758) -, European catfish - *Silurus glanis* LINNAEUS, 1758 -, big eel - *Anguilla anguilla* (Linnaeus, 1758) -, big flounder - *Platichthys flesus* (LINNAEUS, 1758) -, big plaice - *Pleuronectes platessa* LINNAEUS, 1758, the great cormorant - *Phalacrocorax carbo* (LINNAEUS, 1758) -, commonly observed at the Albert Canal, and the great crested grebe - *Podiceps cristatus* (LINNAEUS, 1758) (12). But pike, flounder and plaice are far too rare in the canal to be of great influence. Among the birds, the depth of the canal eliminates the grey heron - *Ardea cinerea* LINNAEUS, 1758 - as a predator. As the round goby has only very recently been recognized as an invasive species in the Albert Canal, no research on its predation has been confirmed yet. We observed, however, that round gobies were successfully used as bait by anglers to catch zander.

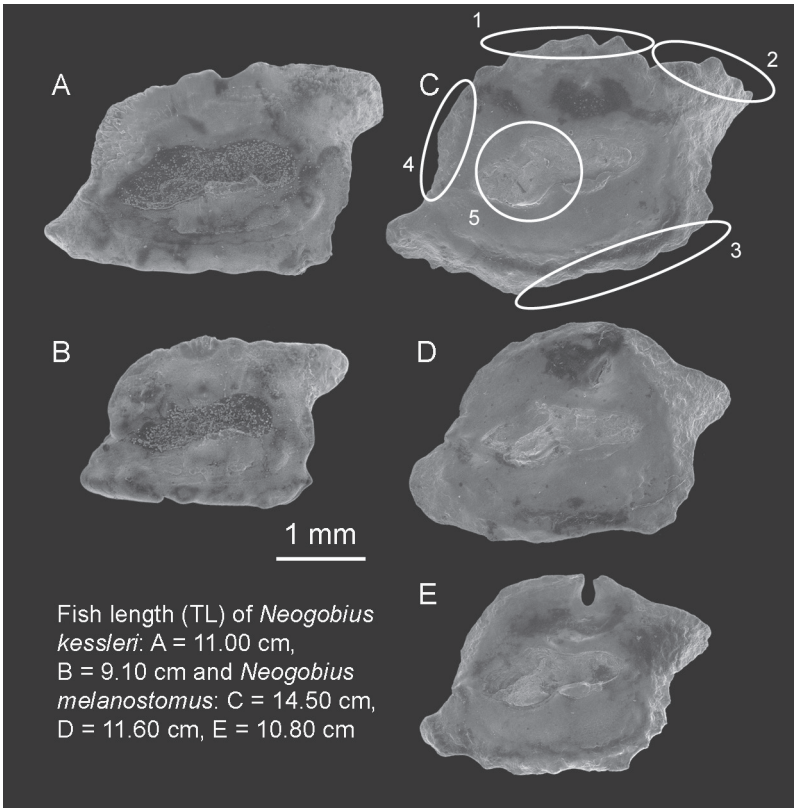

Fig. 3. – Right sagittae of *Neogobius kessleri* (A, B) and *Neogobius melanostomus* (C, D, E). (1) = dorsal rim; (2) = postdorsal process; (3) = ventral rim; (4) = anterior rim; (5) = ostium.

The potentially rapid distribution of the two aforementioned invasive fish species (*N. melanostomus* and *N. kessleri*) can be followed up by direct sampling of river or canal stretches susceptible to yield catches, in particular the mooring places of river barges, where ballast tanks are drained. Another method is the inspection of bird pellets or droppings for fish otoliths along waterways. Predator birds feed on fish, but do not digest their hard parts (such as bones and otoliths), which are subsequently vomited out or ejected in droppings (e.g., 13, 14).

Otoliths (sagittae) provide a useful tool in identifying fishes to species level and they are widely used to that effect in ichthyology, ornithology, cetology and palaeontology (15). For this reason we present here a list of distinctive features (Table 2), and illustrations of the sagittae of *N. melanostomus* and *N. kessleri* (specimens from the RBINS collection, unnumbered, Fig. 3) to enhance identification of otolith finds in pellets of birds. Moreover, using the OL and OH information from Table 2, the length and weight of the consumed fish can be reasonably well estimated. As no specimens of *N. kessleri* from Belgium were available for dissection, we figure otoliths from two specimens caught on the Danube, near Vienna in Austria.

Otoliths of *N. kessleri* and *N. melanostomus* are quite thin in ventral view and have an overall rectangular to parallelogram outline. Those of *N. melanostomus* are characterized by a convex, sometimes notched dorsal rim (feature 1), with the postdorsal process (feature 2) well below the highest point of the dorsal rim. Only the sagittae extracted from a fish of 16 cm TL had a very concave dorsal rim. The otoliths show little marginal ornamentation, except for specimens from larger fish. The posterior part of the ventral rim (feature 3) is bent, whereas the ventral rim in otoliths of *N. kessleri* is more or less straight. The anterior rim (feature 4) is higher in *N. melanostomus* than in *N. kessleri*, due to the convexity of the dorsal rim of the former. The ostium (feature 5, anterior part of the sulcus) is wider in *N. melanostomus* than in *N. kessleri*.

In our small sample, there appears to be a clear relationship, for the larger sizes, between body weight and OL, but less so between TL and OL (Table 1). Two male specimens of 138 mm and two of 140 mm and 143 mm TL resp. have a different OL depending rather on the body weight than on the TL. This contrasts, however, to an earlier study concerning a large sample of the same species from the Baltic Sea, which showed a clear correlation between fish length and body weight (16). Moreover, our sample contains specimens of 105, 110 and 120 mm, represented each by one male and one female. In two cases (TL 110 and 120 mm) the otoliths of the males are larger than those of the females, but in one case (TL 105 mm) the opposite is true. The two largest specimens (one female of 160 mm TL and one male of 157 mm TL) are comparable in size, but the otoliths of the male are much larger than those of the female. Possibly, such differences can be attributed to sexual dimorphism. It must be noted, however, that sexual dimorphism in otoliths is a rarely observed phenomenon and when observed otoliths of male fish are not necessarily larger than those of female fish. It will, however, require much more additional material than presently available to verify our assumptions.

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REFERENCES

1. Verreycken H, Breine JJ, Snoeks J & Belpaire C, 2011. First record of the round goby, *Neogobius melanostomus* (Actinopterygii: Perciformes: Gobiidae) in Belgium. Acta Ichthyologica et Piscatoria 41 (2): 137-140. http://dx.doi.org/10.3750/AIP2011.41.2.11

2. Jacobs P, 2012. Exotische grondelsoorten in het Albertkanaal. Antenne 6 (4): 15-17 [in Dutch].
3. Maitland PS, 1980. *Elseviers gids van de zoetwatervissen*: 230-231. Elsevier, Amsterdam/ Brussel [in Dutch].
4. Kornis MS, Mercado-Silva N & Vander Zanden MJ 2012. Twenty years of invasion: a review of round goby *Neogobius melanostomus* biology, spread and ecological implications. Journal of Fish Biology 80: 235-285. http://dx.doi.org/10.1111/j.1095-8649.2011.03157.x
5. Pinchuk VI & Miller PJ, 2003. *Neogobius melanostomus* (Eichwald, 1831) In: Miller P.J. (ed.), The freshwater fishes of Europe, Volume 8, Part I, *Mugilidae, Ather*In*idae, Ather*In*opsidae, Blenniidae, Odontobutidae, Gobiidae* 1: 173-180, AULA-Verlag, Wiebelsheim, Germany.
6. Corkum LD, Sapota MR, Skora KE, 2004. The round goby, *Neogobius melanostomus,* a fish invader on both sides of the Atlantic Ocean. Biological Invasions 6 (2): 173-181. http://dx.doi.org/10.1023/B:BINV.0000022136.43502.db
7. van Beek GCW, 2006. The round goby *Neogobius melanostomus* first recorded in the Netherlands. *Aquatic* In*vasions* 1 (1): 42-43. http://dx.doi.org/10.3391/ai.2006.1.1.10
8. Kottelat M & Freyhof J, 2007. *Handbook of European Freshwater Fishes*. Cornol, Switzerland and Berlin, Germany.
9. Miller PJ, 1986. Gobiidae. In: Whitehead PJP, Bauchot ML, Hureau JC, Nielsen J & Tortonese E, *Fishes of the North-eastern Atlantic and the Mediterranean*: 1019-1085. UNESCO, Paris.
10. Kirilenko EV & Shemonaev EV, 2012. Feeding of the Round Goby *Neogobius melanostomus* (*Perciformes, Gobiidae*) in Two Volga Reservoirs, Journal of Ichthyology 2012 Vol 52 no 4 pp. 291-295. http://dx.doi.org/10.1134/S0032945212020063
11. Dorenbosch M, 2009. Population increment of native and alien fish species in the Dutch rivers Rhine and Meuse: Competition and relations with environmental variables. In: Lucy FE (ed.), Proceedings of the 16th ICAIS conference, 19-23 April 2009, Montreal, Canada: 127. http://www.icais.org/pdf/2009abstracts/Martijn_Dorenbosch.pdf
12. Bellanger RM & Corkum LD, 2003. Susceptibility of Tethered Round Gobies (*Neogobius melanostomus*) to Predation in Habitats with and Without Shelters, J. Great Lakes Res. 29 (4): pp. 588-593, Internat. Assoc. Great Lakes Res.
13. Leopold MF, van Damme CJG & van der Veer HW, 1998, Diet of cormorants and the impact of cormorant predation on juvenile flatfish in the Dutch Wadden Sea. Journal of Sea Research 40(1-2):93-107.
14. Suter W & Morel P, 1996. Pellet analysis in the assessment of Great Cormorant *Phalacrocorax carbo* diet: reducing biases from otolith wear when reconstructing fish length. Colonial waterbirds 19(2):280-284.
15. Nolf D, 1985. Otolithi Piscium. In: Schultze HP (ed.), *Handbook of Paleoichthyology*, vol. 10: 145 pages. Fischer Verlag, Stuttgart/New York.
16. Sokołowska E & Fey DP, 2011. Age and growth of the round goby *Neogobius melanostomus* in the Gulf of Gdańsk several years after invasion. Is the Baltic Sea a new Promised Land? Journal of Fish Biology 78: 1993-2009.

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