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Shallow-water soft bottom macrozoobenthic communities from Edremit Bay (NE Aegean Sea)

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ABSTRACT. Qualitative and quantitative aspects of macrozoobenthic fauna in Edremit Bay were studied. Benthic samples were collected from 20 stations at depths ranging from 1 to 30m in October 2002. Salinity of sea water varied between 35.3 and 38.6psu, temperature between 18.8 and 21.6ºC, dissolved oxygen between 2.6 and 8.9mg/l, and silt-clay percentage of sediment between 1.6 and 94.1. A total of 139 macrozoobenthic taxa were identified, of which Polychaeta was the dominant group (44.6% of species, 42.7% of specimens). Shannon-Weaver's Diversity Index (H'), Pielou's Evenness Index (J'), Soyer's Frequency Index (F), Bray-Curtis similarity measure and Spearman's rank correlation coefficient were applied to the presence and abundance of the benthic fauna. Medium diversity index values (between 2.7 and 4.8) but high evenness index values (between 0.75 and 0.98) were determined in the area. The cluster and nMDS analysis showed that there were 6 distinct species assemblages in the area. A positive correlation was determined between silt-clay percentage, and number of individuals, number of species and diversity index value. The number of specimen is also positively correlated with depth.

KEY WORDS : Zoobenthos, environmental variables, Edremit Bay, Aegean Sea.

INTRODUCTION

Benthic organisms including a variety of feeding modes can provide a link between substratum and water column predators. They are important food resources especially for demersal fishes. Since most benthic organisms are sessile or sedentary, the analysis of benthic communities gives a tool to determine the dimension of environmental stresses and if those stresses are natural or due to pollutants (POCKLINGTON & WELLS, 1992; PANCUCCI-PAPADOPOULOU et al., 1999).

Macrobenthic communities inhabiting the Greek coasts of the Aegean Sea were intensively studied (ZARKANELLAS & KATTOULAS, 1982; KISELEVA, 1983; ZENETOS et al., 1991; SIMBOURA et al., 1998; PANCUCCI-PAPADOPOULOU et al., 1999). Researches at the Turkish coasts were mainly focused in İzmir Bay (ÖNEN, 1983; ERGEN et al., 1994; ERGEN & ÇİNAR, 1994; ÇİNAR et al., 1998).

Edremit Bay is one of the most important fishery regions of the Aegean Sea. It is located between the longitudes E26º34'34''-E26º56'44'' and latitudes N39º18'41''-N39º34'45''. This region supports dense populations of many demersal fishes and is also suitable for trawling (KOCATAŞ & BILECIK, 1992). However, increasing number of summer houses, light food industry and fisherman ports are major factors threatening the ecosystem of the bay.

The aim of this study is to assess macrozoobenthic species inhabiting different depths of Edremit Bay and their distributional patterns according to major environmental variables. The present study also analyzed the structure and ecological features of macrobenthic communities prevailing in the area where no detailed study has been previously performed on this subject.

MATERIALS AND METHODS

This study was conducted in Edremit Bay in October 2002 (Fig. 1). Macrobenthic samples were obtained by means of a 0.1m^2 Van Veen grab from four transects, namely A, B, C and D, perpendicular to the coast line. The bottom was sampled at five stations corresponding to 1, 5, 10, 20 and 30m depths at each transects. These four transects and five depths at the shores of different human population densities were chosen as representatives of the ecological situation of the bay. Transect A is located at the shore of most dense population throughout the year, transects B and C are at the shores of summer houses and transect D is at the shore of non-settled area. Three replicates were collected at each depth.

All benthic samples were sieved through a 0.5mm mesh sized sieve and then the retained material was fixed with 4% formaldehyde-sea water solution. In the laboratory, macrobenthic organisms were sorted into main taxonomic groups, and then identified to species level and counted.

Small portions of surface sediment were removed from grab samples for analysis of silt-clay percentage (FOLK, 1974). The bottoms of stations are sand or silty-clay, except for B2 and D2, where the bottom was covered with the phanerogames *Cymodocea nodosa* (Ucria) Ascherson and *Posidonia oceanica* (Linnaeus) Delile, respectively.

For the determination of temperature, salinity and dissolved oxygen of sea water, water samples were taken by means of a 3 liter water sampler just above the sea bottom. The temperature was measured by thermometer on the water sampler, salinity by Mohr-Knudsen method (IVANOFF, 1972) and dissolved oxygen by Winkler method (WINKLER, 1888).

Shannon-Weaver's (1949) Diversity Index (H') (log base 2) and Pielou's (1975) Evenness Index (J') were calculated on the basis of the qualitative and quantitative composition of the macrobenthic fauna.

Soyer's (1970) Frequency Index (F) was used to determine the frequencies of species in the study area and results were evaluated as constant (F≥50%), common (50%>F≥25%) and rare (F<25%).

The numerical abundance data obtained per sampling station were analyzed using cluster and non-metric multidimensional scaling (nMDS) techniques, based on the Bray-Curtis similarity (Clarke & Warwick, 2001). Prior to the analysis, the raw data expressed as number of individuals in each sample were transformed using the fourth root transformation. The cluster and nMDS analysis were performed on a previously reduced set of species in order to limit the noise caused by the very rare species (with only one specimen). SIMPER analysis was performed to identify the percentage contribution of each species to the overall similarity within each group that was assessed according to results of the cluster analysis.

In order to determine the correlation between the biotic (species number, specimen number, community diversity, evenness) and abiotic (depth, dissolved oxygen, silt-clay%) parameters, Spearman's rank correlation coefficient was used (Siegel, 1956).

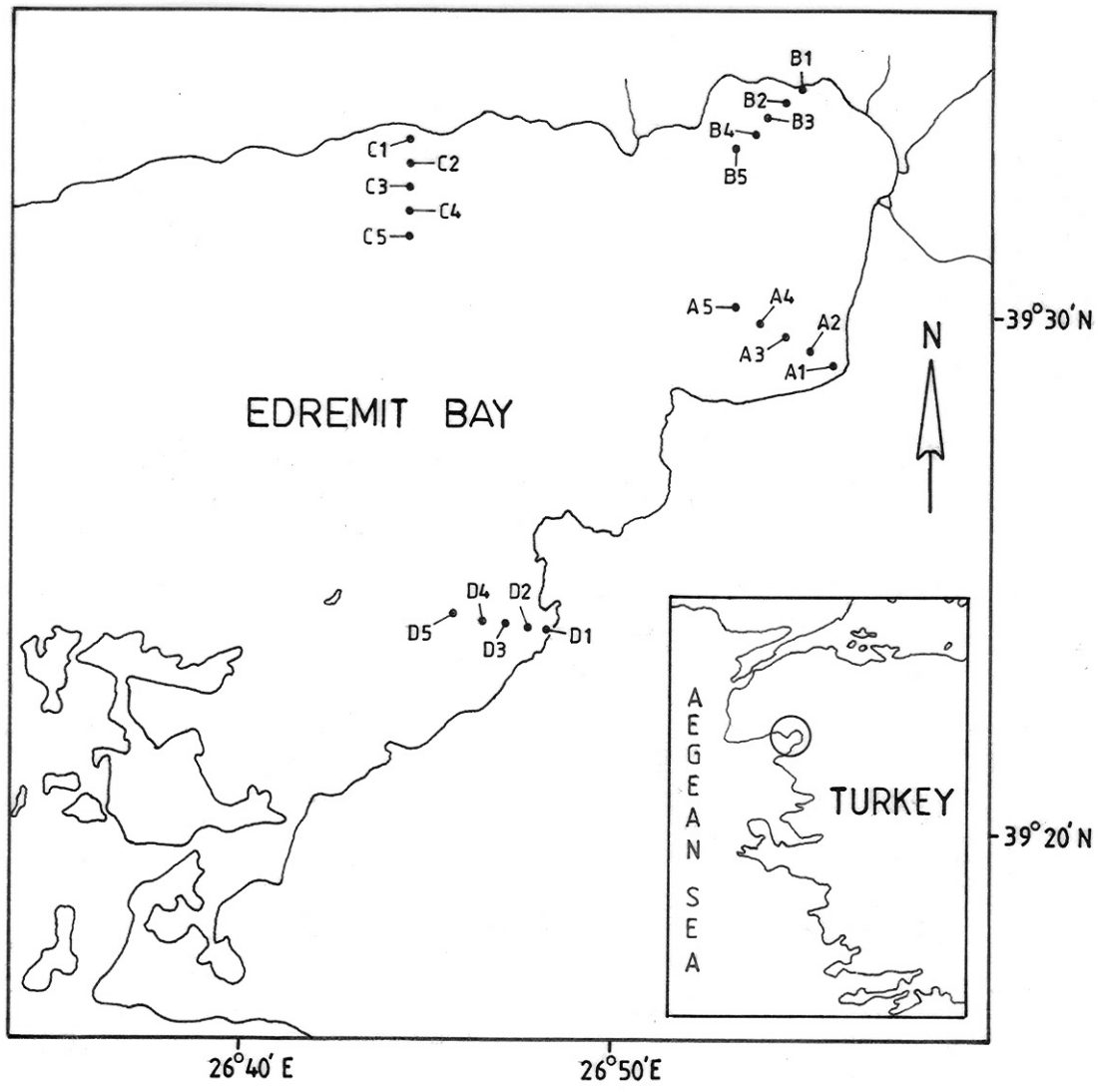

Fig. 1. – Positions of the sampling stations in Edremit Bay.

RESULTS

The difference in sea water salinity values of depths was statistically significant ($p<0.05$). Salinity values differed from 38.2 to 38.6psu at all transects between 1 and 20m depths except C1 (37.7psu), from 37.5 to 37.9psu at 30m depth at all transects except D5 (35.3psu). Temperature values did not vary among depths ($p>0.05$). It varied between 18.8 (B1) and 21.6 (C2) ºC in the area. Dissolved oxygen values of depths differed from 2.6 (B5) to 8.9 (B1) mg/L and did not show a significant statistical difference among depths ($p>0.05$). Depth greater than 20m had lesser dissolved oxygen values than shallower waters except transect C. Silt-clay percentage of sediment varied between 1.6 (D1) and 94.1 (B4) and did show a significant difference among depths ($p<0.05$). It had its minimum values at 1m depth of each station (1.6-3.6%) and extremely increased after 5m depth downward, except station C2 (2.5%).

TABLE 1

Some abiotic and community parameters at each sampling site (S: Salinity, T: Temperature, dO: Dissolved oxygen mg/l, S: Species number, N: Abundance per 1m2, H': Diversity index value, J': Evenness index value)

Site	Depth	S (psu)	T (ºC)	Silt-Clay %	dO	S	N	H'	J'
A1	1	38.6	19.9	3.5	6	9	43	2.9	0.93
A2	5	38.2	19.9	43.5	8	15	250	3.2	0.84
A3	10	38.4	19.5	56.2	6.7	13	260	3.1	0.85
A4	20	38.5	19.1	84.8	5.9	14	160	3.5	0.93
A5	30	37.5	21.2	39.6	4.7	17	270	3.8	0.93
B1	1	38.4	18.8	3.6	8.9	12	63	3.5	0.97
B2	5	38.5	18.9	26	8	43	523	4.7	0.88
B3	10	38.5	19.1	89.4	4.9	21	260	3.3	0.75
B4	20	38.5	21.1	94.1	3.2	17	290	3.8	0.94
B5	30	37.9	21.1	82.6	2.6	13	186	3.6	0.98
C1	1	37.7	21.2	1.8	5.4	9	67	3	0.96
C2	5	38.6	21.6	2.5	4.9	9	80	3	0.97
C3	10	38.5	21.4	56.4	3.2	29	310	4.5	0.92
C4	20	38.4	21.4	76.6	5.6	28	579	4.3	0,90
C5	30	37.9	21.2	41.1	5.6	20	356	4	0.93
D1	1	38.5	20.1	1.6	6.2	12	87	3.2	0.89
D2	5	38.5	19.9	45.7	4.4	39	243	4.8	0.91
D3	10	38.4	19.8	67.4	7.7	21	220	4	0.91
D4	20	38.6	19.9	52.4	4.7	13	167	3.5	0.94
D5	30	35.3	19.9	38.3	4.9	9	127	2.7	0.91

A total of 139 macrozoobenthic taxa were identified during the course of this study. The number of species at stations ranged from 9 to 43 and the number of specimens from 43 to 579 (per $1m^{-2}$). The most abundant taxonomic groups in terms of number of species and number of specimens were Polychaeta (44.6% of species, 42.7% of specimens) and Mollusca (26.6% of species, 37.3% of specimens), respectively.

Half of the total number of taxa (67 species) appeared only once in samples. Among these, Polychaeta was represented by a total of 30 species and was followed by Crustacea (17 species), Mollusca (13 species) and Echinodermata (7 species). Only three species can be classified as constant in the area; *Nephtys hombergii* occurred at 13 stations, *Ampelisca diadema* at 11 stations and *Amphiura chiajei* at 10 stations. Twenty species were common and others rare.

Species represented by high number of individuals in the area were *Nephtys hombergii* (maximum density: $87ind.m^{-2}$), *Corbula gibba* (maximum density: $83ind.m^{-2}$), *Rissoa splendida* (maximum density: $63ind.m^{-2}$), *Notomastus latericeus* (maximum density: $60ind.m^{-2}$), *Nephthys incisa* (maximum density: $57ind.m^{-2}$), *Processa* sp. (maximum density: $50ind.m^{-2}$), *Mellinna palmata* (maximum density: $40ind.m^{-2}$), *Nucula nitidosa* (maximum density: $40ind.m^{-2}$) and *Ampelisca diadema* (maximum density: $37ind.m^{-2}$).

When values of biotic parameters according to depths and transects are examined, it can be seen that the lowest mean value of specimen density per $1m^2$ was found at 1m depth (65±10.4 SE) and transect D (169±32.1 SE), the highest at 20m depth (299±113.1 SE) and transect C (278±106.5 SE). As for the mean number of species the lowest scores were also calculated at 1m depth (11±0.9 SE) and transect A (14±1.5 SE) and the highest at 5m (27±9.8 SE) and transect B (21±6.3 SE). The lowest mean Shannon-Weaver diversity index value was determined at 1m depth (3.1±0.1 SE) and transect A (3.3±0.1 SE), the highest at 5m depth (3.9±0.5 SE) and transects B and C (3.7±0.2 and 0.3 SE). The lowest mean evenness value was calculated at 10m (0.85±0.04 SE) and transect A (0.89±0.02 SE), and the highest at the deepest depth, 30m (0.93±0.01 SE) and transect C (0.93±0.01 SE). The phanerogames *Cymodocea nodosa* and *Posidonia oceanica* found at B2 and D2 (5 m) greatly increased the number of species and diversity index values estimated at 5m. The high values of standard error calculated at this depth were mainly due to the effect of these phanerogames.

The nMDS and cluster analysis (Fig. 2) based on the fourth-root-transformed species abundances in each station revealed six distinct species assemblages in the area.

The high similarity values were estimated within the groups A (64%) and D (64%). The species much contributed to the similarity of the group A according to the SIMPER analysis are *Plagiocardium papillosum* (contribution: 18%), *Gouldia minima* (14%), *Myrtea spinifera* (14%) and *Ampelisca diadema* (13%). The group D including stations A2 and A3 is characterized by high densities of *Corbula gibba* (max. 70ind.m^{-2}), *Nephtys incisa* (max. 57ind.m^{-2}), *Acanthocardia paucicostata* (max. 40ind.m^{-2}) and *Nucula nitidosa* (max. 13ind.m^{-2}). The other associations B, C, E and F as shown on nMDS plot in Fig. 2 have an average similarity of 35%, 46%, 47% and 44%, respectively. The species responsible for the high similarity in the group B are *Lumbrineris gracilis* (% contribution: 11), *Ampelisca diadema* (9%), *Melinna palmata* (9%) and *Corbula gibba* (8%). The group C including the stations A4 and D3 is characterized by the species *Nephtys incisa*, *Gouldia minima*, *Pitar rudis* and *Myrtea spinifera*; the group E including five deepest stations by the species *Nucula nitidosa* (% contribution: 12), *Tellina distorta* (11%) and *Nephtys hombergii* (11%); the group F having four shallow water stations (1m) by *Spisula subtruncata* (26%), *Chamelea gallina* (26%), *Diogenes pugilator* (13%) and *Nephtys hombergii* (11%). There is a high dissimilarity between the groups discerned and the stations C2, C3 and D2. As D2 has a dense *Posidonia oceanica* meadow, a number of species found at this station did not occur at any other stations, resulting in a high dissimilarity between this station and the others. The station C2 had a smaller number of species and some species (*Pelogenia arenosa*, *Caprella acantifera* and *Echinocyamus pusillus*) occurred only at this station. Although C3 possessed relatively high number of species, the high dominance levels of *Processa* sp. (50ind.m^{-2}) and *Microdeutopus stationis* (30ind.m^{-2}) caused dissimilarity between this station and the rest.

According to SIMPER analysis, the average similarity level at each depth was very low, being 44% among samples taken from 1m depth, 15% among samples taken from 5m depth, 24% among samples taken from 10m depth, 28% among samples taken from 20m depth and 31% among samples taken from 30m depth. As it was shown in the dendrogram of Fig. 2, samples taken from 1m has a high faunal affinity. A relatively high similarity (31%) was also calculated among samples collected at 30m. The most contributing species to the similarity of this depth are as follows: *Nephtys hombergii* (22%), *Corbula gibba* (11%), *Processa* sp. (10%) and *Tellina distorta* (10%).

TABLE 2

Spearman rank correlation coefficient (rs) between biotic and abiotic parameters. (dO: dissolved oxygen; NS: Non Significant) n=20

	Number of species	Number of specimens	Community diversity (H')	Evenness (J)
Depth	0.228	0.484	0.257	0.104
	NS	p<0.05	NS	NS
dO	-0.106	-0.172	-0.179	-0.380
	NS	NS	NS	NS
% Silt-Clay	0.529	0.520	0.443	-0.139
	p<0.05	p<0.05	p<0.05	NS

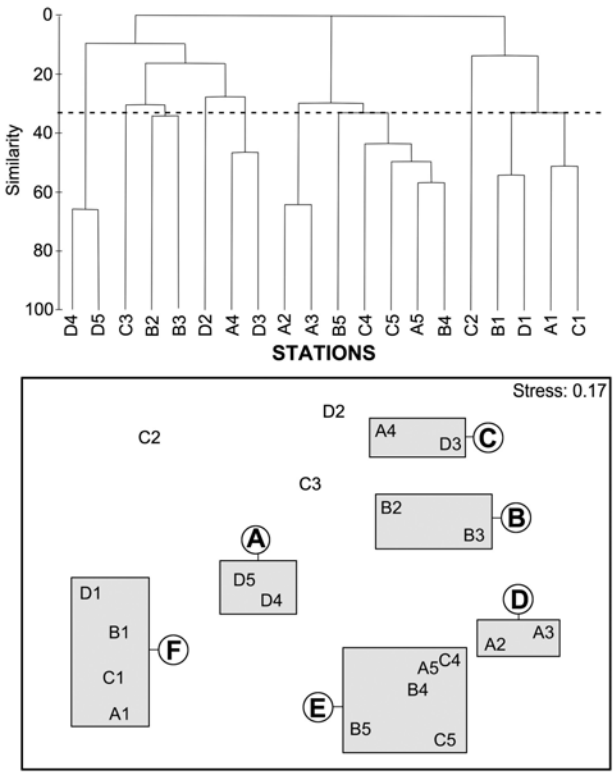

Fig. 2. – Affinity of the sampling sites according to cluster (horizontal line shows 35% similarity level) and non-metric multidimensional sampling analyses.

A positive correlation was determined between silt-clay percentage, and number of individuals, number of species and diversity index value. The number of specimen is also positively correlated with depth (Table 2).

DISCUSSION

Faunistical analyses of macrozoobenthic fauna on the soft substratum of Edremit Bay revealed a rich species composition. The highest numbers of species were encountered at stations B2 (43 species) and D2 (39 species). These stations differed from the others in having phanerogames; *Cymodocea nodosa* formed dense meadows at B2 and *Posidonia oceanica* at D2. These phanerogames are known to provide suitable microhabitats and niches for a variety of organisms in the Mediterranean Sea (PERES, 1967; MAZZELLA et al., 1989; ÇİNAR et al., 1998; SÁNCHEZ-JEREZ et al., 1999; ÇİNAR, 2003a). A total of 30 species that occurred in these phanerogames were not found in other stations that possessed sand or silt-clay bottom structure. These species are previously reported from the same habitats in the Mediterranean Sea (PERES, 1967; MAZZELLA et al., 1989; ÇİNAR et al., 1998).

As seen from cluster analysis, except for samples taken from 1m depth, there was no high similarity among the samples taken from the same depths, showing that the area has a heterogeneous bottom structure and different

hydrodynamic conditions that greatly influence the distribution of the species. One-way ANOVA showed that the mean percentage of silt-clay of each assemblage shown in Fig. 2 is statistically significant ($p<0.05$). For example, the group F contained samples taken from 1m, which was characterized by having a low percentage of silt-clay (mean: 2.6±0.54 SE), whereas the group C, which included samples from 10 and 20m, had the highest silt-clay percentage (mean: 76.1±8.7 SE). A high similarity (average score: 44%) calculated among samples collected at 1m depth. The samples were characterized by the presence and low densities of the species *Spisula subtruncata*, *Chamelea gallina*, *Diogenes pugilator* and *Nepthys hombergii*. Having a low silt-clay percentage showed that relatively strong hydrodynamic forces occur at 1m depth and consequently much affect the community structure at this depth than those at the other depths. The substrate characteristics and hydrodynamic forces were considered as one of the principal factors governing the community structures in the shallow water benthic environments of the Aegean Sea (SIMBOURA et al., 1995).

The Aegean Sea is considered as oligotrophic (MIHALATOU & MOUSTAKA-GOUNI, 2002), and such areas have low diversity index but high evenness index values (PANCUCCI-PAPADOPOULOU et al., 1999). Samples taken in this study presented generally medium community diversity values whereas evenness values were high. Similar results were reported from South Evvoikos Gulf (NW Aegean Sea) by SIMBOURA et al. (1998), but higher diversity index values from Rhodes Island (SE Aegean Sea) by PANCUCCI-PAPADOPOULOU et al. (1999). Moreover, ERGEN & ÇINAR (1994) also determined higher diversity index values at the south-eastern Aegean Sea in comparison to the northern part of the Aegean Sea. PANCUCCI-PAPADOPOULOU et al. (1999) attribute this situation to the prevailing current regime sediment sorting and subtropical character of the southern Aegean Sea.

A positive correlation was determined between the number of individuals and depth ($p<0.05$). ZENETOS et al. (1991) and PANCUCCI-PAPADOPOULOU et al. (1999) reported a negative correlation between depth, and species number and abundance. However, ZENETOS et al. (1991) collected materials from 75-200m depths and indicated that the highest number of species occurred at the shallowest depth (75m) and the lowest at the deepest one (200m), whereas PANCUCCI-PAPADOPOULOU et al. (1999) obtained materials from 30-400m depths and they observed higher values of biotic variables mainly at depths between 30-70m. In addition, MACKIE et al. (1997) and ÇINAR (2003b) concluded that the nature of substratum (sediment composition) and depth were the major environmental variables influencing the distribution of polychaetes from the southern Irish Sea and northern Cyprus, respectively. ÇINAR (2003b) studied syllid compositions of habitats from 0 to 600m along the northern Cyprus and stated that the number of syllid species and individuals, and diversity and evenness index values were negatively correlated with depths. In our study, the mean number of species attained its maximum level at 5m because of the presence of the phanerogames and then gradually decreased to 30m depth. However, the mean diversity index values are fairly constant among depths.

Among the species encountered in this study, the polychaetes *Lumbrineris latreilli* and *Notomastus latericeus*, the amphipod *Ampelisca diadema*, and the bivalves *Nucula nitidosa* and *Myrtea spinifera* are known to be typical species of fine or mixed sediments; the polychaete *Melinna palmata*, and the bivalves *Acanthocardia paucicostata* and *Corbula gibba* are characteristic species of muddy bottoms (ZARKANELLAS & KATTOULAS, 1982; POPPE & GOTO, 1993; SIMBOURA et al., 1998). All of these species were generally represented by high number of individuals at the stations.

The macrobenthic community structure identified during this study indicates that a relatively undisturbed condition prevails in the area. A total of four stations had a diversity value between 4.1 and 5, twelve stations between 3.1 and 4, and four stations between 2.7 and 3. Soft bottom benthic habitats can be classified based on community diversity index as bad ($0<H'\leq1.5$), poor ($1.5<H'\leq3$), moderate ($3<H'\leq4$), good ($4<H'\leq5$) and high ($H'>5$) (SIMBOURA & ZENETOS, 2002). According to the above classification, 20% of the stations are in good condition, 60% in moderate and only 20% in poor condition. However, some polychaetes, which are considered as indicator species of semi-polluted or transitional zones of the Mediterranean Sea when they form high-density populations, such as *Lumbrineris gracilis, L. latreilli* and *Monticellina heterochaeta* (PEARSON & ROSENBERG, 1978; SIMBOURA et al., 1998; ERGEN et al. 2002), exist in the area. These species were frequently found at stations but generally no high abundance levels of these species were encountered. The bivalve *Corbula gibba* can attain high abundance at the periphery of the severely polluted environment (PEARSON & ROSENBERG, 1978; ZARKANELLAS & KATTOULAS, 1982). This species was represented by high number of individuals in the study and particularly dominated the stations A2, A3, B3 and B4, together with the other tolerant species *Lumbrineris gracilis*. Diversity index values also were lower than four at these stations. Other stations of transect A were partly dominated by species *Lumbrineris gracilis*, *Notomastus latericeus* and *Abra alba*, which can survive in organically polluted bottoms. Moreover, the lowest mean Shannon-Weaver diversity index value was determined within this transect. The above mentioned stations are located at the innermost region of the bay and seem to be affected by increasing human settlements and the food industry in the area.

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Life History and Biology of *Diloba caeruleocephala* (Figure of Eight) (Lepidoptera ; Noctuidae)

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ABSTRACT. *Diloba caeruleocephala* (L.) is an important pest in fruit-growing areas of Turkey where its hosts include almond, apple, pear, plum, peach, apricot, and cherry. We studied the life history of this species using *Amygdalus communis* L. (Rosacae) as its host at 26ºC, 60±5% RH, with 16: 8 (L: D) photoperiod and fluorescent lighting in the laboratory. Their eggs are laid in clusters on shoots of the host and hatch in about 6 days. The life cycle from egg to adult requires 27-35 days. Adults mate 3-4 days after emergence, and females begin laying eggs 3 days later.

KEY WORDS : *Diloba caeruleocephala*, Figure of Eight, Moth, Noctuidae

INTRODUCTION

Diloba caeruleocephala (L.) (Lepidoptera: Noctuidae: Dilobinae) is a polyphagus pest, with almond, apple, pear, plum, peach, apricot, and cherry among its most important hosts. First instars attack newly opened buds of the host, while older instars feed along the main leaf veins. Damage to fruit was documented by MAÇAN (1986). BODENHEIMER (1958) discussed the importance of this species to fruit trees in Turkey.

Diloba caeruleocephala is reported from all Europe, Lebanon, Israel, North Africa, the Russian states of federation and Asia (CAYROL, 1972 ; DOLLMAN, 1958 ; MÜLLER, 1953 ; POPOV, 1962). MAÇAN (1986) reported it from the southeastern and eastern Anatolia region of Turkey ; and KANSU (1995) and NIZAMLIOĞLU (1961) documented its distribution to Ankara and Istanbul.

Because of its considerable economic importance and given that details of the life history are poorly documented, we studied the biology of this species in the laboratory. The results are presented in this paper.

MATERIALS AND METHODS

Diloba caeruleocephala adults (n=50) were captured in the vicinity of Diyarbakır in the autumn of 2002. Eggs were obtained by placing the females in a screen cage (40x30x30cm) with 15cm lengths of *Amygdalus communis* L. shoots. Newly laid eggs were removed daily, counted, and kept in petri dishes (10cm diameter) on moist filter paper. Larvae were fed with freshly cut host-plant leaves and the larvae were transferred to new leaves every second day. The development of larvae was observed daily, and shed larval head capsules were collected, measured and preserved in 70% ethyl alcohol. Pupae were harvested daily and transferred to a new cage (40x30cm) containing a potted host plant. The larvae and pupae were weighed individually, and pupal length was also measured. The colony was maintained under controlled laboratory conditions at 26ºC with 16: 8 (L: D) photoperiod. All data were subjected to analysis of variance (ANOVA), and means were separated using Fisher's Least Significant Difference (LSD) ($P<0.05$).

RESULTS

Eggs

The eggs were laid in clusters on the upper surface of the host shoot, leaves or on the cage (Fig. 1A). The number of eggs in each cluster varied from as few as 12 to as many as 155. The egg clusters are covered with hair-like scales left by the female moth (Fig. 1B). The eggs are elliptical and light green to brownish, about 0.81±0.02mm (mean ± SD) in length and 1.00±0.02mm in width (n=25) with a flattened base and slight depression at the micropyle (Fig. 1C-D). They are sculptured with 13-16 vertical raised ridges (Fig. 1C). Incubation requires 5.8±0.4 days at 26ºC, and the colour of the egg changes from light green to brownish yellow at about 4 days.

Larvae

Larvae develop through five instars. Larval weight, length, and head capsule measurements (n=15) in each instar are shown in Table 1.

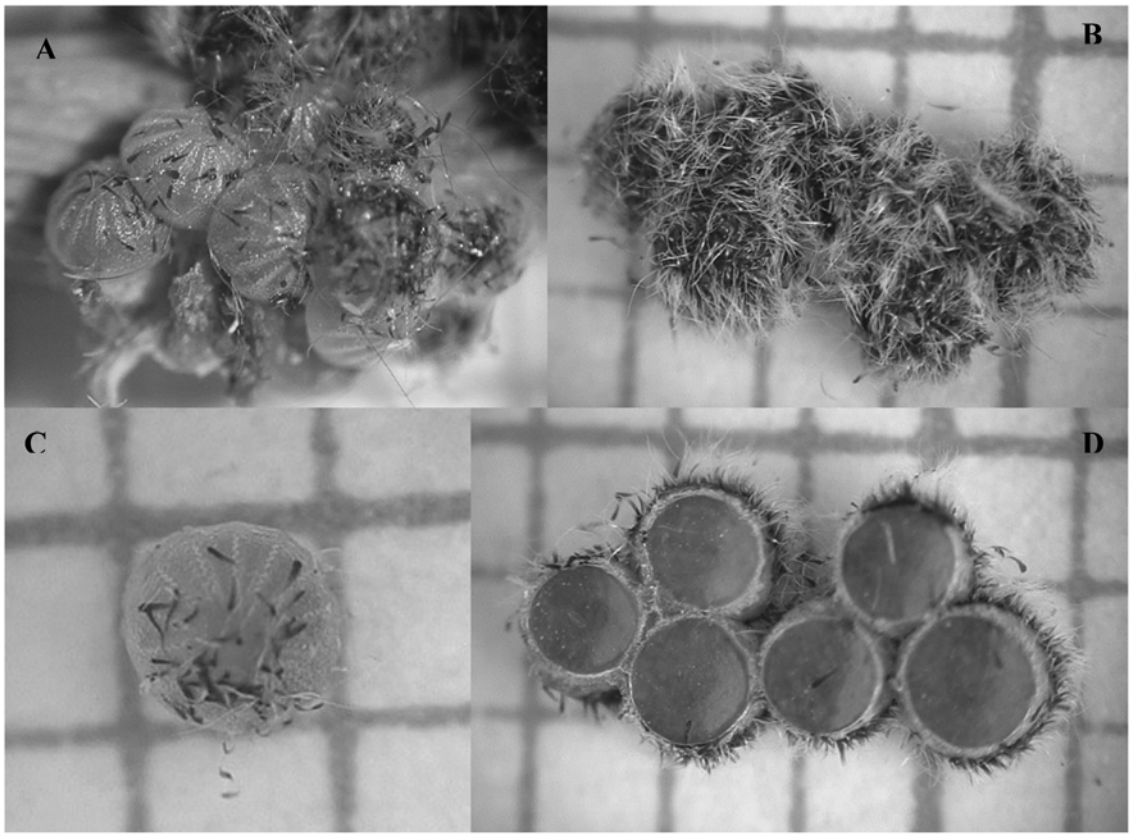

Fig. 1. – A composite photo of egg stages of *Diloba caeruleocephala* (Scale=4X)

TABLE 1

Measurements of head capsule length, weight, and length of each larval instar of *Diloba caeruleocephala* (n=15) and their developmental times in days (n=25)

Instar	Head capsule measurements (mm)	Weight (mg)	Length (mm)	Development duration (days)
First	0.35±0.01 e	4.2±1.0 e	2.2±0.9 e	4.1±0.6
Second	0.68±0.01 d	11.5±3.9 d	7.1±0.5 d	4.2±0.7
Third	0.81±0.01 c	21.3±4.3 c	14.4±1.4 c	4.5±0.3
Fourth	1.44±0.01 b	46.8±5.5 b	22.2±1.2 b	4.0±0.5
Fifth	1.95±0.01 a	65.3±8.2 a	38.2±0.9 a	4.1±0.6

Means within a column followed by a different letter are different (P<0.05) (Fisher's Least Significant Difference tests).

The first instar is light brown to dark brown, with long setae over the body (Fig. 2). The head capsule in the first instar has many long setae brownish in colour with two large brown patches. The legs and prolegs are light brown and tarsal segments were black to brown. The anal prolegs are dark brown. Antennae are black, with dark black basal area. The larvae eat their eggshells and aggregate on the underside of the leaf, typically spinning some silk web on the leaf. They chew a small amount of leaf tissue on the underside of the leaf, creating a small pit, which is gradually enlarged as the larvae continue to feed.

Fig. 2. – The first instar of *Diloba caeruleocephala* (Scale=4X)

The second instar is black with yellow subdorsal bands. Each segment has a row of short, branching small spines. The head is cream with two black dorsal stripes extending posteriorly (Fig. 3).

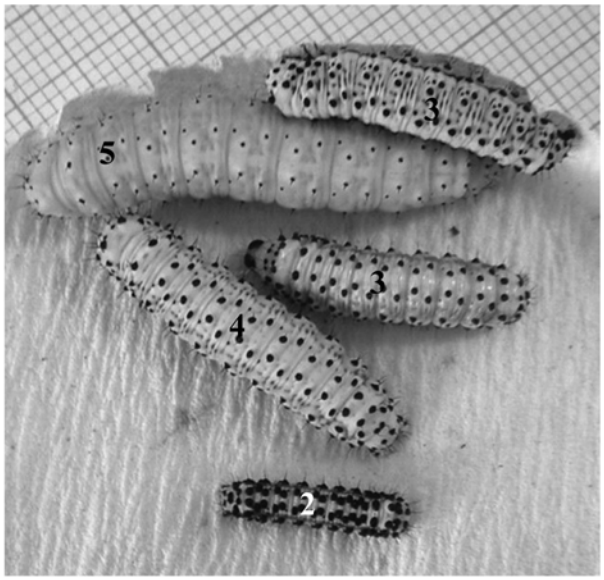

Fig. 3. – The second (2), third (3), fourth (4) and fifth (5) instar stages of *Diloba caeruleocephala* (Scale=1X)

The mouthparts are black. The integument is textured with black spots (Fig. 3). The longitudinal, dorsal and subdorsal bands are more evident in the second instar than in the first instar. The thoracic legs are black with the tarsal claws darkened.

The third instar differs in appearance to the second instar but has similar black patches on the head capsule. The body is grayish blue with yellow dorsal and subdorsal bands (Fig. 3). The head is grayish blue with two large black patches. The third instar generally rests on the sides of leaves and feeds on leaf edges. The fourth instar is similar in appearance to the third instar. The body of the fifth instar is light green with light yellow dorsal and subdorsal bands (Fig. 3).

Pupae

Pupae are initially soft and light tan in colour with speckled black and brown markings (Fig. 4).

The 10th segment of the pupa bears the cremaster by which the pupa attaches to a substrate. The pupae are in cocoons made of soil or leaf debris (Fig. 5). They measured 17.4±0.5mm in length, 0.6±0.2mm in width and weighed 21 to 42mg (average 32.1±7.0mg, n=25). The duration of the pupal stage is 6.2±1.2 days.

Adults

Males and females are similar in appearance (Fig. 6) but differ in their size and antennae. The male's antennae have a double comb, whereas the female's antennae are thin and long.

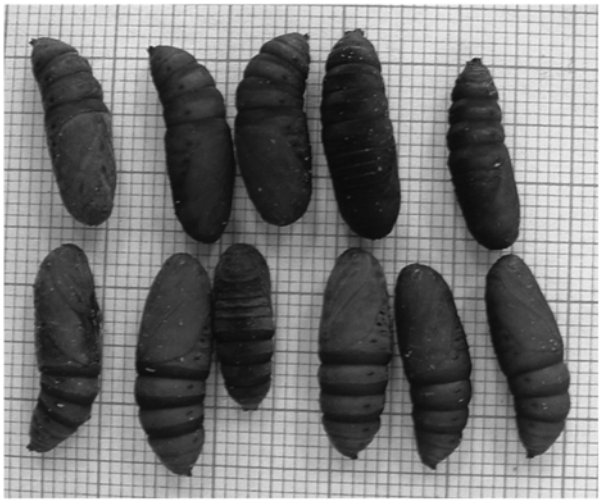

Fig. 4. – The pupae of *Diloba caeruleocephala* (Scale=1X)

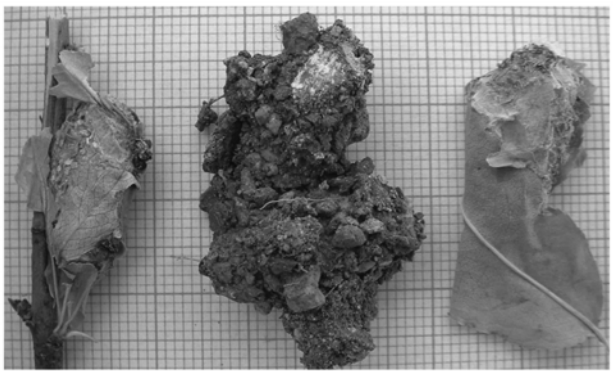

Fig. 5. – The cocoons of *Diloba caeruleocephala* (Scale=1X)

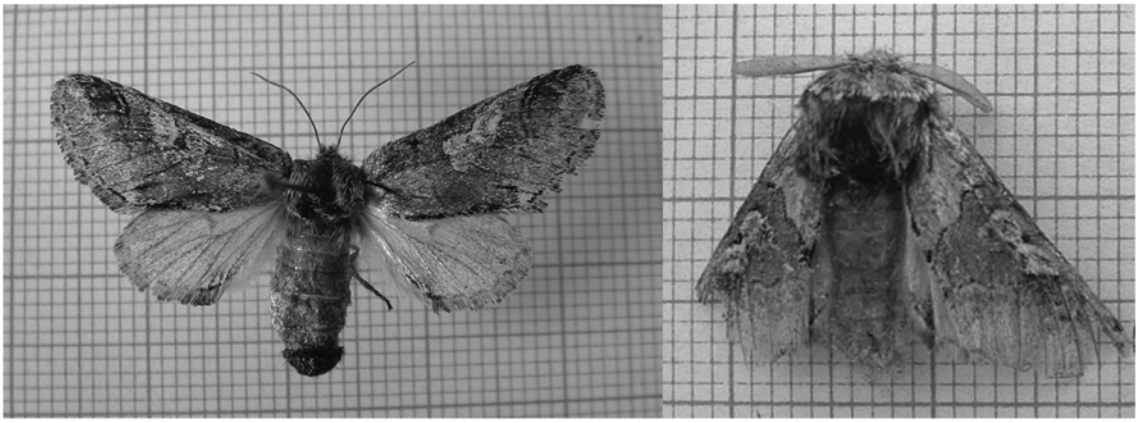

Fig. 6. – The female and male of *Diloba caeruleocephala* (Scale=1X)

The wingspan is 38±0.4mm in females and 34±0.5mm in males (n=25). Mating pairs often rest together for 4-6 hours. Females started laying eggs about 3 days after mating and each laid from 130-174 eggs (n=25). Adults survived about 2 weeks in the laboratory.

The duration from egg to adult emergence is 27-35 days at 26ºC, 60±5% RH, 16: 8 (L: D) photoperiod in the laboratory.

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Ant biodiversity conservation in Belgian calcareous grasslands: active management is vital

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ABSTRACT. A list of ant species collected in eight calcareous grasslands in the Viroin valley (Viroinval, Belgium) is presented. Thirty species were identified, including *Temnothorax albipennis*, for the first time recorded in Belgium. Ant community composition and chorology of some ant species are discussed. Recommendations on how to use ant community composition and nest densities of several ant species to evaluate management in calcareous grasslands are given. It appears that in locations with encroachment of tall grasses (especially *Brachypodium pinnatum*) and spontaneous afforestation, due to a complete lack of or to inadequate management, most of the often rare xerophilic ant species are replaced by mesophilic, rather common species.

KEY WORDS : ant biodiversity conservation, calcareous grassland, nature management, Belgium

ABSTRACT. Un inventaire des fourmis collectées à l'aide de pièges d'activité dans huit pelouses calcicoles de la vallée du Viroin (Viroinval, Belgique) est présenté. Trente espèces ont été identifiées dont une, *Temnothorax albipennis*, est signalée pour la première fois de Belgique. Des remarques sont données sur les communautés de fourmis et leur composition spécifique, ainsi que sur la chorologie de certaines espèces. Des suggestions sont émises à propos de la prise en compte des communautés de certaines espèces de fourmis et des densités de leurs nids dans le cadre de l'évaluation des effets de la gestion des pelouses calcicoles. A cet égard, nous pensons que dans les sites où le tapis graminéen (comme le *Brachypodium pinnatum*) se densifie ou que la recolonisation préforestière s'amorce, la plupart des espèces xérothermophiles, souvent rares et très localisées, seront remplacées par des éléments plus mésophiles, largement plus répandus et communs.

INTRODUCTION

The calcareous grasslands of northwestern Europe comprise the dry grasslands on limestone and chalk as well as those on calcareous loess. These grasslands originated after felling of primordial forest, which had already started in prehistoric times. Since these habitats are seminatural, they require some form of management by grazing or mowing to prevent afforestation. They were once widespread in the hilly calcareous regions of Europe, but because of changes in agricultural practice their extent has decreased dramatically. Since World War II urbanisation, abandonment of grazing practice and fertilisation resulted in a dramatic decrease of these grasslands in Belgium. Everywhere in Europe this habitat type is severely pressured by threats that impinge upon several ecological communities. A shift in land use is one of the most important among these. Moreover the remaining areas are often strongly fragmented. This poses threats of extinction and inbreeding for the remaining characteristic arthropod populations (WallisDeVries et al., 2002; Vandewoestijne et al., 2005).

Calcareous grasslands, especially the ones on dry slopes and plateaus, are known as being rich in plant diversity and they often contain many rare species (Mitchley & Xofis, 2005). Regions which have this rich floristic diversity are often biodiversity hot spots for a wide variety of invertebrates. In Belgium the calcareous grasslands of the Viroin valley are such habitats (Adam et al., 1991; Honnay et al., 2004; Butaye et al., 2005a,b). They are catalogued as a Belgian hot spot for several groups, i.e. Heteroptera (Delescaille et al., 1991), Saltatoria (Decleer et al., 2000), butterflies (Goffart & De Bast, 2000; Vandewoestijne et al., 2005), bees and wasps (Claessens, 1992; Longo et al., 1992) and spiders (Bara, 1991).

In Belgium the ant fauna of these habitats is rather poorly investigated. Elsewhere in Europe calcareous grasslands and other similar thermophilic grasslands are known to have a rich ant fauna often including very rare species (Mabelis, 1983; Gallé, 1986; Seifert, 1996; Meyer-Hozak, 2000; Braschler & Baur, 2003; 2005; Ottonetti et al., 2006). In the northern part of the country (Flanders), calcareous grasslands are scarce and the one that was recently well studied at the Sint-Pietersberg, was indeed a hot spot of ant biodiversity (more than 25 species) in Flanders (Dekoninck et al., 2003; 2005). In the southern part of Belgium (Wallonia), calcareous grasslands are more widespread but their ant fauna is

poorly known. GASPAR (1965; 1971) investigated the habitat type in the Famenne region, whilst the Viroin valley has been studied by LEMAL (1978) and later by DELESCAILLE et al. (1991). But others have never been sampled for their ant fauna. Recently, two localities in the Viroin valley, respectively at Treignes and Vaucelles, were studied by BAUGNÉE (2004). He recorded *Camponotus piceus* (Leach, 1825) for the first time in Belgium and suggested that this interesting region still has a lot of entomological "secrets" to reveal. Hence, when a new pitfall sampling campaign on xeromorphic and rocky soils (*Xerobromion*=XB and *Mesobromion*=MB) in the Viroin valley was started, rare, extinct or new species for the Belgian ant fauna were expected. As different nature management types, microhabitats and vegetations were sampled, this study also enables us to evaluate the influence of the type or the lack of management in these grasslands on the occurring ant communities.

The main aim of the present inventory is to investigate if there is a specific ant fauna in the typical xeromorphic, rocky and calcareous grasslands of the Viroin valley and to give a complete list of all known ant species of this region. We also want to essay the possibilities to use ant community composition and the occurrence and abundance of several typical ant species to evaluate the effects of management and to make some remarks on the importance of active management for ant community composition of calcareous grasslands in general.

MATERIALS AND METHODS

Study area

The Viroin valley is located in southwestern Belgium, mainly in the municipality of Viroinval (province of Namur). The Viroin, a river of medium size (about 25km long and 5-10m width) flows between a succession of hills and joins with the Meuse near Givet in the French Ardennes. The geological formation dates from the Devonian period and occurs as limestone hills ('Tiennes' in French) in the landscape. In the Viroin valley two major types of calcareous grasslands are present: mesomorphic calcareous grasslands with five different plant communities, and xeromorphic grassland with three different plant communities (see HONNAY et al., 2004; BUTAYE et al., 2005a,b). The mesomorphic calcareous grasslands in the Viroin valley belong to the *Mesobromion* and show an intermediate position between the Central European and Atlantic calcareous grassland communities (BUTAYE et al., 2005a). The xeromorphic calcareous grassland communities on the other hand show strong affinities with the calcareous vegetation of Central Europe and southern Europe. The Viroin valley is a favourite region for entomologists and other naturalists because some of the most notable Belgian calcareous grasslands occur there (DUVIGNEAUD et al., 1990).

TABLE 1

Codes, locality, description, management, exposition, distance to other habitat and area of the sampled localities (with temperature: +=cold, ++=warm, +++=hot sites)

Code	Locatity (city)	Vegetation and description site	Temperature	Management	Exposition	Distance to other habitat	Area
TdL(WO)	Tienne du Lion (Frasnes)	Open woodland: *Pinus nigra nigra*, *Brachypodium pinnatum*, *Teucrium chamaedrys*, mosses on NW slope.	+	None	South-East	20m open grassland	10.000m²
Cha(XB)	Chalaine (Nismes)	*Xerobromion*: xeromorphic site with a lot of stones and bare ground; vegetation: *Teucrium chamaedrys*, *Galium pumilum*, *Anthyllis vulneraria*, *Thymus praecox*, *Helianthemum nummularium* and *Ononis repens*.	+++	Short intensive summer grazing	South	40m forest and arable field	2.500m²
FdC(XB)	Fondry des Chiens (Nismes)	*Xerobromion*: south-exposed slope with stones and a scarce vegetation, a sharp slope, with vegetation similar to Cha(XB).	+++	None	South	100m forest	4.500m²
RTr(MB-XB)	Roche Trouée (Nismes)	*Meso-Xerobromion*: south-exposed slope with grasses (*Brachypodium pinnatum* and *Festuca ovina*) but also other typical calcareous herbs.	++	Short intensive summer grazing	South	20-25m forest	2.500m²
TaP(XB)	Tienne aux Pauquis (Dourbes)	*Xerobromion*: a lot of stones, short grassland on a sharp slope, *Origanum vulgare*, *Sedum* sp., *Teucrium chamaedrys* and *Prunella lanciniata.*	+++	None	South-West	25m forest	2.000m²
TaP(MB)	Tienne aux Pauquis (Dourbes)	*Mesobromion*: uniform grass layer (80%) surrounded by *Buxus-Carpinus betulus* forest, regularly chopped.	+	None (regularly chopping)	South-West	5-10m forest	6.000m²
TdR(MB-XB)	Tienne des Rivelottes (Treignes)	*Meso- Xerobromion*: straight, south-exposed slope, with *Gymnadenia conopsea*, *Sanguisorba minor*, *Cirsium acaule*, *Potentilla neumanniana*, *Helleborus foetidus* and *Scabiosa columbaria.*	+++	Mowing in September	South	25m forest	8.750m²
Aba(MB)	Les Abannets (Nismes)	*Mesobromion* with well developed *Brachypodium pinnatum* layer.	++	None	South	20m forest	7.500m²

TABLE 2

Vegetation characteristics at each site (given in percentage ground cover or height in cm) (TdL=Tienne du Lion; Cha=Chalaine; FdC=Fondry des Chiens; RTr=Roche Trouée; TaP=Tienne aux Pauquis; TdR=Tienne des Rivelottes and Aba=Les Abannets).

Code site	Stones %	Trees %	Shrubs %	Herbs %	Grasses %	Mosses %	Veg Height (cm)	Bare ground %	*Brachypodium pinnatum* %	*Festuca ovina*
TdL(WO)	5	15	15	10-15	80	50	30-40	0	75	0
Chat(XB)	20	0	0	80	15	20	15-20	10	5	10
FdC(XB)	40	0	15	75	20	30	15	5	0	20
RTr(MB-XB)	5	0	5	30	60	40	30	5	20	20
TaP(XB)	45	0	10	20	40	20	20	25	5	40
TaP(MB)	0	0	10	35	80	5	35	5	70	5
TdR(MB-XB)	10-15	0	10	30	40	5	30	5	20	20
Aba(MB)	0	0	20	45	90	5	45	0	80	0

In this study, eight localities were sampled for ants (see Table 1 and 2). They are all included in part of the nature reserve of the Viroin (for details, see http: //mrw.wallonie.be/dgrne/sibw).

Sampling and identification of ants

In all investigated grasslands three pitfall traps were installed. Pitfalls with a diameter of 9,5cm were placed in a row, spaced 3-5m apart. A 3,5% formaldehyde solution was used as a fixative and detergent was added to lower surface tension. Pitfalls were emptied monthly. Sampling lasted from September 2002 until October 2003.

Although the use of pitfall trapping to collect ants has been questioned by some (e.g. SEIFERT, 1990), in many investigations capture rates are used to estimate the relative abundance of particular species in simultaneously sampled sites, i.o.w. to assess the habitat preference of these species (RETANA & CERDÁ, 2000; SCHLICK-STEINER et al., 2005; STEINER et al., 2005; BOTES et al., 2006; OTTONETTI et al., 2006).

All species were morphometrically investigated and identified using SEIFERT (1996). Some specimens were inspected by B. Seifert and two gynes of *Lasius jensi* were deposited in the collection at the Staatliches Museum für Naturkunde of Görlitz. All other voucher specimens were deposited in the personal collection of the first author.

Implications and possibilities of pitfall sampling for ant community studies

Capture rates of pitfall traps not only depend on population densities (abundance) of the species caught, but also on intra- and interspecific differences in bottom surface activity levels and in trapping efficiency as influenced by habitat structure (GREENSLADE, 1964, MAELFAIT & BAERT, 1975, BAARS, 1979, HALSALL & WRATTEN, 1988; ANTVOGEL & BONN, 2001; BONTE et al., 2003). Therefore, they are not suited to compare different species in their abundance. If, however, resulting from a long enough sampling period in not too structurally different sampling sites, they give good estimates of the relative abundance of each particular species over the sampling sites (MAELFAIT & BAERT, 1975; BAARS, 1979; DESENDER & MAELFAIT, 1986; MAELFAIT, 1996). When used for ordinations capture rates therefore have to be transformed giving each species equal weight, what we did hereafter in the program PC-ORD (MC CUNE & MEFFORD, 1999; MC CUNE & GRACE, 2002). An indirect gradient analysis, Detrended Correspondence Analysis (DCA) was carried out. Only the most abundantly caught species were used here. DCA-ordinations are interpreted against the background of the available dataset of environmental variables, yielding a biplot, based on the 'a posteriori'-obtained significant correlation values between DCA axes and environmental variables.

Indicator Species Analysis

Characteristic species for different types of calcareous grasslands or different management regimes were explored by Indicator Species Analysis (DUFRÊNE & LEGENDRE, 1997). An Indicator Species Analysis is a useful method to find indicator species and/or species assemblages characterising groups of samples. This analysis gives information on the concentration of species abundance in a particular group of samples and on the faithfulness of occurrence of a species in this particular group. Indicator values are tested on their statistical significance using a randomization (Monte Carlo) technique. *A posteriori* groups that were tested here are the ones obtained by DCA analysis and the groups of pitfalls with the same management regime. Species with a significant IndVal higher than 50% are considered as 'indicators' i.e. characteristic and/or typical for a group of pitfalls.

Characterisation of the environmental variables

At each of the eight sampling sites the most abundant plant species were noted and the habitat structure determining variables such as dominant grasses (percentage ground cover of *Festuca ovina* and *Brachypodium pinnatum*), cover of other vegetation layers (herbs, trees, mosses, bare ground, the amount of stones and rocks and vegetation height were estimated (see Table 2). High abundance of *B. pinnatum* is often used as an indicator of calcareous grassland habitat deterioration and is a negative indicator of habitat quality and condition (BOBBINK & WILLEMS, 1987; 1988; 1991; BOBBINK et al., 1988; BUTAYE et al., 2005a,b; MITCHLEY & XOFIS, 2005). Also temperature conditions, exposition, total habitat area and distance to other habitat were noted (see Table 1).

Management

In most calcareous grasslands in the Viroin valley, management measures for biodiversity conservation are of recent date. Only in three sites a continuous management is carried out (Table 1). In Roche Trouée and Chalaine both situated in Nismes, short intensive summer grazing is used to control tall grass encroachment and consists of 20-25 sheep that are put in a 25 x 25m mobile raster "as long as needed". In Tienne de Rivelottes the management regime during the last years was mowing in September.

RESULTS

Faunistics and general results

During the study 30 different species were found (37.5% of the Belgian Formicidae (DEKONINCK et al., 2006)). *Temnothorax albipennis* was recorded for the first time in Belgium. The ant fauna composition of the different sites is presented in Table 3. *Lasius niger* (Linnaeus, 1758) the most common Belgian ant, was not found during the project. Two species were found in each of the

TABLE 3

Species composition at the different sites (x=only found in isolated specimen or very small numbers, xx=abundant, xxx=very abundant) with Belgian status (CO=common ant species, RA=rare and VR=very rare ant species) and between () their habitat preference (Ca=calcareous grasslands and rocky habitats, Xe=xeromorphic grasslands and other hot habitats, Wo=woodlands, Bo=bogs, He=heathlands), (TdL=Tienne du Lion; Cha=Chalaine; FdC=Fondry des Chiens; RTr=Roche Trouée; TaP=Tienne aux Pauquis; TdR=Tienne des Rivelottes and Aba=Les Abannets).

Species	Used codes	Status (habitat preference)	Location							
			TdL (WO)	Cha (XB)	FdC (XB)	RTr (MB-XB)	TaP (XB)	TaP (MB)	TdR (MB-XB)	Aba (MB)
Aphaenogaster subterranea (Latreille, 1798)	APHASUBT	RA (Ca)			xxx				x	
Formica cunicularia (Latreille, 1802)	FORCCUNI	CO		x	x	x	xx		x	
Formica fusca Linnaeus, 1758	FORCFUSC	CO	x			x	xx	x		
Formica lusatica Seifert, 1997	FORCLUSA	RA (Xe)				x				
Formica pratensis Retzius, 1783	FORCPRAT	RA (Xe)	x							x
Formica rufibarbis Fabricius, 1798	FORCRUFI	RA (Xe)		x		x	x			
Formica sanguinea Latreille, 1798	FORCSANG	CO (Xe, He)		x						
Lasius alienus Förster, 1850	LASIALIE	CO (Ca)	x	xxx	xxx	xxx	xxx	x	xxx	xx
Lasius flavus (Fabricius, 1781)	LASIFLAV	CO		xx	x	x	x	x	x	x
Lasius fuliginosus (Latreille, 1798)	LASIFULI	CO	x	x						x
Lasius jensi Seifert, 1986	LASIJENS	VR (Ca)			x				x	
Lasius mixtus (Nylander, 1846)	LASIMIXT	CO	x	x	x	x	x		x	
Lasius platythorax Seifert, 1991	LASIPLAT	CO (Wo, Bo)	xxx			xx	x	x		xxx
Lasius sabularum (Bondroit, 1918)	LASISABU	RA				x				
Lasius umbratus (Nylander, 1846)	LASIUMBR	CO							x	
Leptothorax acervorum (Fabricius, 1793)	LEPTACER	CO	x							
Temnothorax albipennis (Curtis, 1854)	TEMNALBI	RA (Ca)				x				
Temnothorax interruptus (Schenck, 1852)	TEMNINTE	RA (Ca)		xx	x		x		x	
Temnothorax nylanderi (Förster, 1850)	TEMNNYLA	CO (Wo)					x		x	
Temnothorax unifasciatus (Latreille, 1798)	TEMNUNIF	RA (Ca)					x			
Myrmica rubra Linnaeus, 1758	MYMIRUBR	CO	x	x						
Myrmica ruginodis Nylander, 1846	MYMIRUGI	CO (Wo)	xx							x
Myrmica sabuleti Meinert, 1860	MYMISABU	CO	xx	x	xxx	xxx	xxx	xxx	x	x
Myrmica scabrinodis Nylander, 1846	MYMISCAB	CO	x	x	x	x	xx		x	x
Myrmica schencki Emery, 1894	MYMISCHE	RA (Xe)	x	xxx			x			
Myrmecina graminicola (Latreille, 1802)	MYRMGRAM	(RA) under recorded		x	x	xxx	x		x	x
Ponera coarctata (Latreille, 1802)	PONECOAR	RA (Xe)		x		x				
Stenamma debile (Förster, 1850)	STENDEBI	CO (Wo)	x						x	
Tapinoma erraticum (Latreille, 1798)	TAPIERRA	CO (Ca)	xx	x	xxx	xx	x	xx	xx	xx
Tetramorium impurum (Förster, 1850)	TETRIMPU	CO		x	x		xx	xx		
number of species			**14**	**16**	**12**	**15**	**16**	**7**	**14**	**10**

eight sites in high abundances: *Lasius alienus* and *Tapinoma erraticum*. The ant species richness per site is not significantly correlated with any of the habitat variables, although there is a nearly significant negative correlation with *Brachypodium pinnatum* cover (r=-0.6486, p=0.082).

Status of the collected species

On the basis of conservation status (common, rare, very rare) and habitat choice in Belgium, several groups can be discerned in our species list (Table 3). First we have common Belgian ant species with no preference for any type of habitat (CO in Table 3). We can consider them as additional species in calcareous grasslands. We found 18 of these common ant species (58% of the total).

We also found 12 rare and very rare species (marked with RA and VR in Table 3) restricted in their occurrence to one habitat type (Table 3). Almost all of them have a preference for xeromorphic or rocky habitats in calcareous grasslands. They can be considered as typical species for these grasslands as they are only very exceptionally observed outside these xeromorphic habitats. The observations of *Lasius jensi* are the 3rd and 4th record of this species in Belgium (DEKONINCK & VANKERKHOVEN, 2001). This species, strictly bound to this particular habitat (SEIFERT, 1996), is very rare and threatened in our country. In general the number of rare and very rare species per site is positively correlated with percentage ground cover of *Festuca ovina* (r=0.71 p<0.05) and negatively with *Brachypodium pinnatum* cover (r=-0.86 p<0.05).

Community analysis and correlations with environmental variables

For the indirect gradient analysis only the 14 most abundantly caught species were used. The DCA diagram (Axis 1 and 2) is shown in Fig. 1. Ordination of the sites reveals three groups (Fig. 1). Tienne du Lion and Les Abannets belong to Group 1. The latter is a dense grassland, the other an open woodland with a dense undergrowth, both lacking patches of bare ground and surfacing rocks i.e. they can be characterised as open woodland and as a degraded *Mesobromion* (Table 1). The *Brachypodium pinnatum* cover of these sites is more than 75%. They show a typical ant fauna for woodlands and woodland edges with few thermophilic species. All sites of Group 2 and 3 are *Meso-* and/or *Xerobromions* (MB or XB) with surfacing rocks and stony debris, patches of bare ground and high insolation. The small patches of dense vegetation occurring there are composed of calcareous herbs, not grasses. The sites from Group 2 and 3 (on the left of Fig. 1) have higher average species richness (13.4±3.4) than the sites from Group 1 (12.0±1.0).

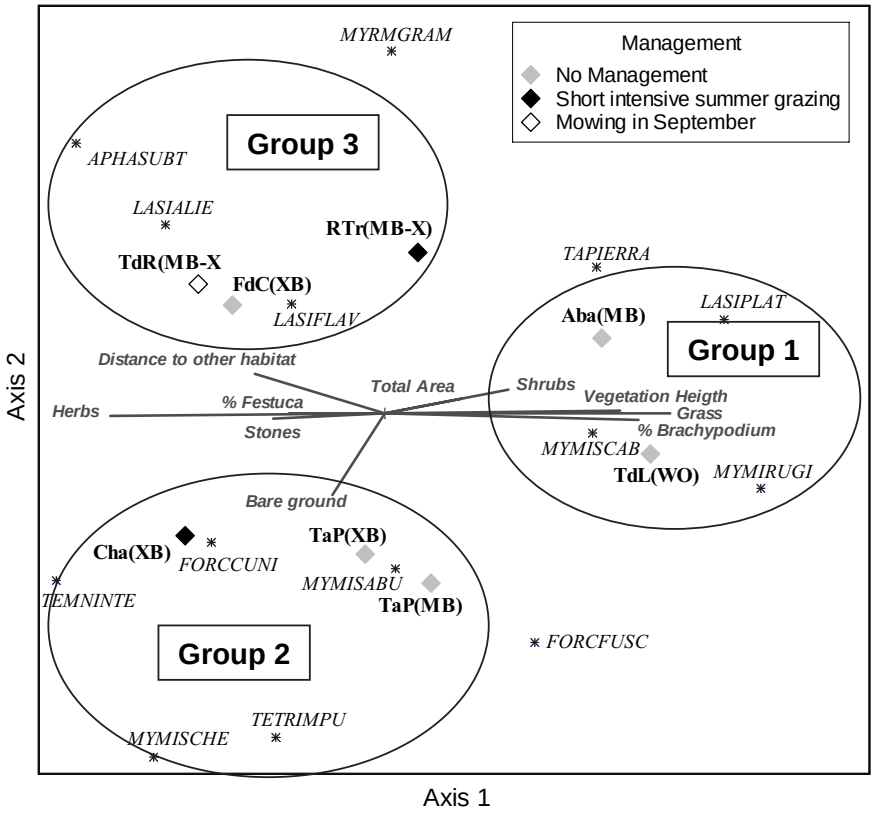

Fig. 1. – Detrended Correspondence Analysis (DCA) diagram (Axis 1 and 2) with 14 most dominant species (percentages explained on Axis 1=58% and on Axis 2=22%). Species are presented with * and their codes (see Table 3) and the sites presented with ◆ in a different colour according to their management (TdL=Tienne du Lion; Cha=Chalaine; FdC=Fondry des Chiens; RTr=Roche Trouée; TaP=Tienne aux Pauquis; TdR=Tienne des Rivelottes and Aba=Les Abannets and WO=woodland, MB=*Mesobromion* and XB=*Xerobromion*).

The abundances of two species are significantly positively correlated with the first axis: *Lasius platythorax* (r=0.882, p<0.01) and *Myrmica ruginodis* (r=0.748 p<0.05). This axis is also positively correlated with the cover of grasses (r=0.901, p<0.01), cover of *Brachypodium pinnatum* (r=0.848 p<0.01) and with average vegetation height (r=0.817, p<0.05). Axis 1 is significantly negatively correlated with the cover of herbs (r=-0.883, p<0.01) and the abundance of *Lasius alienus* (r=-0.813, p<0.05). No species or environmental variable is significantly correlated with the second axis. We can conclude that the main differences in species composition are explained by the gradient going from xeromorphic towards mesomorphic environmental characteristics as was also found by BUTAYE et al., 2005a for plants.

Indicator species

Indicator species with a significant (p<0.05) IndVal higher than 50, were searched for in *a posteriori* groups of pitfalls. Indicator species for a group of pitfalls with the same management regime (3 regimes: No management, Short intensive summer grazing and mowing in September) and for the groups obtained by DCA (Group 1, 2 and 3) are presented in Table 4. For Group 1 from the DCA, five common less thermophilic ant species typical for forest, have a significant IndVal higher than 50: *Formica pratensis*, *Myrmica ruginodis*, *Lasius platythorax*, *Lasius fuliginosis* and *Leptothorax acervorum*. For the *Xerobromiums* and *Mesobromiums* we can distinguish a group (Group 2) with two common (*Myrmica sabuleti* and *Tetramorium impurum*) and one rare xeromorphic species (*Myrmica schencki*) as indicator species and a second group indicator species (for Group 3) with mostly rare and very rare xeromorphic ant species as *Aphaenogaster subterannea*, *Lasius jensi* and *Myrmecina graminicola* and the common ant *Lasius mixtus*.

For groups of pitfalls with the same management regime we also found indicator species. *Lasius platythorax*, an indicator for the less thermophilic ant species group, seems also an indicator for no management activity. Seven rare and xeromorphic species also appear to react positively to short intensive summer grazing management. *Lasius jensi*, *Lasius umbratus*, *Aphaenogaster subterranea*, *Stenamme debile* and *Temnothorax nylanderi* have a significant IndVal higher than 50 for the management regime mowing in September. However as this management regime was only present in one site, these results have to be interpreted with caution.

DISCUSSION

The Viroin valley: a hot spot for ant biodiversity in Belgium?

During this study 30 ant species were recorded. If some other neighbouring and not calcareous grassland localities (for example anthropogenic localities, dense forests, …) would have been sampled too, this list would have even been longer, e.g. *Lasius brunneus* (Latreille, 1798) and *Lasius niger* are very common and widespread species in their typical habitats in this region. *Temnothorax nigriceps* Mayr, 1855; *Solenopsis fugax* (Latreille, 1798); *Camponotus ligniperda* (Latreille, 1802) and *Lasius emarginatus* (Olivier, 1792) are also known for the region, but their colonies are only locally found (LEMAL, 1978; DELESCAILLE et al. 1991). Some other ant species were only recently found in the Viroin region (BAUGNÉE, 2002; 2004): *Plagiolepis vindobonensis* Lomnicki, 1925; *Camponotus piceus* and *Temnothorax parvulus* (Schenck, 1852). Until now, a total of 40 ant species is known from the region. This is almost half of the Belgian ant fauna (DEKONINCK et al., 2006). Hence, the region can be considered as a hot spot for ant biodiversity in Belgium.

TABLE 4

Indicatorspecies with a significant IndVal (IV) 50 (p<0.05), considered as 'indicators' typical for a group of pitfalls with the same management regime (3 regimes: No management, Short intensive summer grazing and mowing in September) and for the groups obtained by DCA (Group 1, 2 and 3)

Species	IV	p	Management
Lasius platythorax	64	0.03	No management
Formcica lusatica	50	0.03	Intensive summer grazing
Lasius sabularum	50	0.03	Intensive summer grazing
Temnothorax albipennis	50	0.03	Intensive summer grazing
Lasius flavus	65	0.02	Intensive summer grazing
Myrmecina graminicola	87	0.01	Intensive summer grazing
Formica rufibarbis	89	0.01	Intensive summer grazing
Ponera coarctata	100	0.01	Intensive summer grazing
Aphaenogaster subterranea	58	0.03	Mowing September
Lasius jensi	82	0.02	Mowing September
Stenamma debile	83	0.01	Mowing September
Temnothorax nylanderi	83	0.02	Mowing September
Lasius umbratus	100	0.02	Mowing September
Species	**IV**	**p**	**Group DCA**
Leptothorax acervorum	50	0.02	1
Lasius fuliginosis	75	0.01	1
Lasius platythorax	91	0.01	1
Formica pratensis	100	0.01	1
Myrmica ruginodis	100	0.01	1
Myrmica schencki	57	0.05	2
Myrmica sabuleti	66	0.03	2
Tetramorium impurum	94	0.01	2
Lasius mixtus	55	0.01	3
Aphaenogaster subterranea	67	0.01	3
Lasius jensi	67	0.01	3
Myrmecina graminicola	72	0.01	3

Ant communities in calcareous grasslands in the Viroin valley

Lasius niger, one of the most common Belgian ants, was not found during the sampling campaign. In the xero- and mesomorphic grassland sites studied here, this eurytopic ant species is replaced by another, less common *Lasius* s. str. species: *Lasius alienus*. Like *L. alienus*, also *Tapinoma erraticum* is found on each of the sites in high abundance. Both species can be considered as common for the region. They are typical for calcareous and other rocky habitats in Wallonia and are almost not present in Flanders (BOER et al., 2003; DEKONINCK et al., 2003; 2005).

In calcareous habitats as the ones studied here, a lot of *Formica* species are found. The *Serviformica* species, *Formica lusatica*; *Formica cunicularia* and *Formica rufibarbis* are found in the open xeromorphic vegetations and are probably frequently used as slaves by *Formica sanguinea*. In less thermophilic sites the *Serviformica* species *Formica fusca* is probably often used to start the nests of the wood ant *Formica pratensis* (a *Formica* s. str. species). Although most *Formica* and *Serviformica* species have a rather wide foraging range and are sometimes found far from their nesting sites (BRASCHLER & BAUR, 2003), we suggest, as their occurrence on a particular site can rapidly be assessed by visual inspection, they can be used in evaluation and monitoring campaigns as indicators for a good management regime. Especially when a rapid evaluation of a site is needed the presence of one or several of these species is indicative for special micro-climatological conditions and high conservation value of the site.

Also within the genera *Myrmica*, *Temnothorax* and *Leptothorax* with small-sized species and the other *Myrmecinae* species we can clearly distinguish ant groups preferring specific temperature conditions (see Fig. 1; Table 3 and 4). Sites can be dominated by indifferent, less thermophilic ant species from woodlands and closed grassland vegetations, occurring here on places with a high ground cover of trees and of the grass *Brachypodium pinnatum*: *Myrmica ruginodis*, *Myrmica rubra*, *Myrmica scabrinodis, Stenamma debile* and *Leptothorax acervorum*. When tree and tall grass cover decrease and the site becomes a more thermophilic habitat, species from this group are replaced by *Tetramorium impurum*, *Myrmica sabuleti* and *Myrmica schencki* and with further decrease (as in the well-developed *Xerobromion* grasslands) by species as *Myrmecina graminicola*, *Aphaenogaster subterranea* and *Temnothorax interruptus*.

To detect changes in ant communities caused by changing management regime, pitfall trapping will be needed, because most of these species cannot be identified with certainty in the field (SEIFERT, 1988a; 1996). Moreover, the chance to detect their presence (small nests usually in low densities) by visual inspection in the field is very low. We recommend a monitoring by three to six pitfall traps (during 6 months May-October) in these sites where management regimes are changed or will be implemented.

The occurrence and abundance of *Lasius* s.str., and especially of a lot of *Chthonolasius* species can also be a good measure for the special temperature and vegetation conditions of well developed calcareous grassland and hence the nature value of particular sites. Their identification in the field is even more difficult (SEIFERT, 1988b; 1992; 1996) than the identification of most *Myrmecinae* species but in contrast to the latter, *Lasius* s. str. and even *Chthonolasius* spec. can be more easily collected in the field and their nest densities can be determined after identification of nest samples in the laboratory. Special attention should be given to the exact nest localities and local conditions of some of the rare *Chthonolasius* species. Also mixed nests could help us to reveal the host preference of these parasites (DEKONINCK et al., 2004; LEHOUCK et al., 2004). As is the case for the *Myrmecinae* species, also *Lasius* s.str. and *Chthonolasius* have characteristic species for different types of calcareous grasslands. Changes in their nest species composition, foraging abundance (to be detected with pitfall traps) and nest density (field observation and hand collecting) can be used in the evaluation of management measures.

In general, where encroachment of dominant grasses (*Brachypodium pinnatum*) and litter accumulation have already taken place in calcareous grasslands in the Viroin valley, the abundance of xerophilic, typical calcareous grassland species is lower than the abundance of less thermophilic, more common species. Species from more nutrient-rich grasslands can be expected to invade these neglected calcareous grasslands. When nests of thermophilic ant species decrease in number and are replaced by nests of species from the indifferent group, part of the very valuable ant fauna, characteristic for the region, will be lost.

Monitoring ant communities in calcareous grasslands?

Some of the studied calcareous grasslands in the Viroin region probably contain the richest ant faunas in Belgium and house a lot of very rare species. So management and monitoring is needed to conserve these very important ant communities. Calcareous grasslands are predominantly semi-natural habitats and they require some form of management by grazing or mowing. Here, different species or species groups can have conflicting demands as long-term experiments on the management of calcareous grasslands have clearly shown that very often different taxonomic groups react in a different way on the respective management treatments (BRAUCKMANN et al., 1997; WALLISDEVRIES et al., 2002). Habitat quality is often diminished by the encroachment of dominant grasses (*Brachypodium pinnatum*) and litter accumulation (BUTAYE et al., 2005a) due to a lack of active management, too low intensity management and probably also by eutrophication through aerial deposition (WALLISDEVRIES et al., 2002).

At present two sites lack management and continue evolving towards shrub and tree vegetations with high abundances of *B. pinnatum*. As we saw, two species are significantly positively correlated with that type of vegetation change: *Lasius platythorax* and *Myrmica ruginodis* Monitoring by pitfall traps and mapping nests of these two ant species could be an early warning system to assess the effects of management or of the lack of it. High abundance of the very competitive grass *B. pinnatum* is mostly caused by insufficient management (BOBBINK & WILLEMS, 1987; 1991; BUTAYE et al., 2005a,b) and increasing nutrient deposition (BOBBINK & WILLEMS, 1988) it leads to a dramatic decrease of the nature value of calcareous grassland (BOBBINK & WILLEMS, 1987; 1991; MITCHLEY & XOFIS, 2005).

Sometimes very contradictory views can be found in literature on the effects of grazing management in general or particular forms of it on the biodiversity of grasslands (BESTELMEYER & WIENS, 2001; BOULTON et al., 2005), and calcareous grasslands in particular (FISCHER et al., 1996; DUTOIT & ALARD, 1997; BARBARO et al., 2001; DAUBER & WOLTERS, 2005; MITCHLEY & XOFIS, 2005). Here only short intensive summer grazing with sheep and no grazing management can be evaluated. Under sheep

grazing conditions the vegetation in *Mesobromions* occurs as a short, tight turf with an extremely high plant species richness and diversity turnover at a strikingly small scale (BUTAYE et al., 2005a). In such situations, grazing can indeed favour ant communities from the thermophilic group. When mesomorphic calcareous grasslands are abandoned or management is lacking, their stability is disrupted and *B. pinnatum* starts to dominate resulting in higher vegetation and large amounts of litter. Both processes cause a reduction of the light penetration to the ground, resulting in a decrease in the competitive ability and finally to local extinction of typically xerophilic plant species (BUTAYE et al., 2005a) and, as we see here, the ant species bound to that kind of vegetation. In *Xerobromions* the regional climate is often modified on a local scale by the influence of topography. Inclination and exposition determine the openness of the vegetation, but when shifting towards more mesomorphic environments, grazing by sheep or rabbits becomes increasingly important.

Not only in this particular region, but also elsewhere in Belgium and Europe, it is likely that in calcareous grasslands with an increasingly dense and high grass layer due to a lack of or an inadequate management, endangered xerophilic ant species will be replaced be mesophilic, rather common species. As management to restore the former open xeromorphic grasslands sites by mowing, grazing and selective clearing started only recently in the Viroin valley, two types of ant monitoring can be very useful. Regularly mapping the nests of *Formica* species and typical xerophilic ant species that can be recognised in the field as *Aphaenogaster subterranean* and *Temnothorax interruptus* and also monitoring by pitfall trapping (3-6 pitfalls during 6 months May-October) for all xeromorphic and rare species that can not be recognized in the field, can be good instruments to evaluate management effects (BRASCHLER & BAUR, 2003; 2005; ENGLISCH et al., 2005). This may also give us more insights in the habitat preferences of the many rare ant species occurring there. We therefore recommend starting an ant monitoring of these grasslands of European importance.

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Measures of the developmental stability, body size and body condition in the black-striped mouse (*Apodemus agrarius*) as indicators of a disturbed environment in northern Serbia

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ABSTRACT. In the present study, an additional combination of end-points was used to evaluate the effects of industrial development on the natural population of the black-striped mouse (*Apodemus agrarius*), previously estimated using cytogenetic and morphometric assays. Developmental stability was assessed by determining the level of fluctuating asymmetry (FA) and the total amount of phenotypic variability (PV). Body weight (BW) and body length (BL) were also used as indices of body size, while the quotient between observed and expected values of body weight presented an index of body condition (BCI). The experimental design employed in this study is unique because only a few studies have been conducted with *A. agrarius* and because no investigator has previously used this combination of end-points in an environmental quality monitoring study. FA was increased in the polluted area for foramen parietalis, foramen dentale, foramen palatinum and foramen angularis. Males from the polluted site had significantly higher FA for foramen parietalis compared with male mice from the reference area. Juvenile animals from the polluted area had significantly higher FAs for foramen dentale and foramen palatinum compared to adults. However, when all the foramina were considered together, a three-way analysis of variance revealed that there was no significant interaction between the factors (sex x age category x site) on the FA (average value). A comparison of PVs indicated higher values in the polluted area for all analyzed characters. For foramen parietalis and foramen dentale, PVs were significantly greater in the polluted site than in the control area. Within the same sex, PVs were significantly greater in mice from the polluted site compared with mice from the control area. The results also indicated that mice trapped at the contaminated site had a reduced body size and poorer body condition. Adult mice exhibited better body condition than juveniles, as revealed by the significantly higher BCI values. Finally, to investigate the relative importance of the three potential biomarkers, a multivariate analysis of variance (MANOVA procedure) was performed. The MANOVA's results revealed the significant effects of age category, site and interaction for sex x site on FA (average value), size and body condition. In conclusion, the results indicated that despite *A. agrarius's* high tolerance to contaminants it may be an important species for environmental quality evaluation studies.

KEY WORDS : body size, body condition, developmental stability, fluctuating asymmetry, foramina.

INTRODUCTION

Typically, the processes of evaluating effects of polluted environments on natural populations of small mammals have been based on a single type of end-point (e.g. cytogenetic, morphometric or morpho-physiological techniques).

Mice from the genus *Apodemus* have been shown to be relevant pollution bioindicators (Berry, 1975; Abramsson-Zetterberg et al., 1997; Stopka & McDonald, 1999; Adrian et al., 2002; Damek-Poprawa, 2003; Ieardi et al., 2003; Metcheva et al., 2001; Topashka-Ancheva et al., 2003). The consequences of such exposure on the biology of animals have been assessed using cytogenetic assays, such as: micronuclei frequencies (Abramsson-Zetterberg et al., 1997; Ieardi et al., 2003), chromosomal aberrations frequencies (Metcheva et al., 2001; Topashka-Ancheva et al., 2003; Velickovic, 2004) and sperm abnormalities (Ieardi et al., 2003) or morpho-physiological assays, such as: morphological (Adrian et al., 2002; Damek-Poprawa, 2003), morphometric (Berry, 1975; Nunes et al., 2001b; Stopka & McDonald, 1999; Velickovic, 2004) and hematological parameters (Nunes et al., 2001a; Topashka-Ancheva et al., 2003).

In the present study an additional combination of endpoints was used to evaluate the effects of industrial development on the natural population of the black-striped mouse (*Apodemus agrarius,* Pallas 1771) previously estimated using cytogenetic and morphometric assays (Velickovic, 2004). Here, besides measuring developmental stability (DS), I also examined the consequences of disturbed environment on body size and body condition.

Developmental stability (DS), (Mather, 1953; Thoday, 1955; or "developmental homeostasis" Lerner, 1954) refers to the ability of an individual to produce a consistent phenotype in a given environment (Graham et al., 1993). Reduced DS can result from wide variety of environmental and/or genetic perturbations (Valentine & Soulé, 1973; Valentine et al., 1973; Siegel & Doyle, 1975a; 1975b; 1975c; Siegel et al., 1992; Yablokov, 1986; Clarke, 1992; 1993).

Fluctuating asymmetry (FA), directional asymmetry (DA) and antisymmetry (AS) are the three recognized types of asymmetry in morphological traits. Each is characterized by a different combination of the mean and variance of the distribution of (R & L) differences (Van Valen, 1962; Palmer & Strobeck, 1986; Palmer, 1994), where R and L represent the measurement of the

right and left sides of bilaterally symmetrical traits respectively.

FA is characterized by normally distributed differences about a mean of zero (VAN VALEN, 1962; LEARY & ALLENDORF, 1989; PARSONS, 1990). MOLLER & SWADDLE (1997) indicated that the metrics for conducting fluctuating asymmetry analysis are derived from taking the difference between right and left traits of bilaterally symmetric organisms and that "they are simple and straightforward to measure, and can be statistically very powerful at detecting differences among populations".

DA is characterized by a normally distributed (R-L) where the mean departs significantly from zero. AS is associated with a bimodal distribution of (R-L) about a mean of zero or more subtly as a broad peaked unimodal (platykurtic) distribution (PALMER & STROBECK, 1986).

Fluctuating asymmetry (VAN VALEN, 1962), where differences in the development of the two sides of a bilaterally symmetrical character are random (PALMER and STROBECK, 1986), has been proposed as an indicator of environmental as well as genetic stress (LEARY & ALLENDORF, 1989; CLARKE, 1992; PARSONS, 1992; MARKOW, 1995). Stress is considered to be a significant and lasting deviation from favourable conditions that leads to abnormal demands and the destabilization of vital processes (LARCHER, 2000).

Furthermore, FA has been extensively used as a measure of developmental instability (DI, the inability of a bilateral organ or organism to buffer its development against disturbances and to produce a predetermined phenotype), MØLLER & SWADDLE (1997). DI, measured as FA, is expected to be positively related to stress and negatively to fitness. Interestingly, the level of fluctuating asymmetry in morphometric characters has been successfully used as a measure of DI in house mouse (*Mus musculus*, Linnaeus 1758) hybrid zones (ALIBERT et al., 1994; ALIBERT et al., 1997; CHATTI et al., 1999).

The total amount of phenotypic variability (PV), (SOULÉ, 1982; PANKAKOSKI et al., 1987; ZAKHAROV, 1987) can be used as a measure of DI when changes in developmental "noise" are responsible for changes in the total phenotypic variability of the population (PANKAKOSKI et al., 1987). According to HALLGRIMSSON et al. (2002), developmental stability and canalization (the ability of a structure to develop along an ideal developmental trajectory under a variety of different environmental conditions; WADDINGTON, 1940) are patterns of phenotypic variability. "Both DS and canalization reflect the tendency for development to follow a preferred trajectory toward some phenotypic outcome", HALLGRÝMSSON et al. (2002).

Several studies have shown that animal species display developmental instability (DI) under various stress conditions, reflected in increased FA and PV, observed in laboratory experiments as well as in natural populations (BAILIT et al., 1970; VALENTINE & SOULÉ, 1973; VALENTINE et al., 1973; AMES et al., 1979; SIEGEL & DOYLE, 1975a; SIEGEL & DOYLE, 1975b; SIEGEL & DOYLE, 1975c; ZAKHAROV, 1981; ZAKHAROV, 1984; PANKAKOSKI et al., 1987; ZAKHAROV et al., 1991; STUB et al, 2004; SØRENSEN et al., 2005). Increased environmental stress can therefore be expected to lead to an increased breakdown of homeostatic mechanisms, resulting in increased FA and phenotypic variability (VØLLESTAD et al., 1998).

Body size is the most obvious and fundamental characteristic of an organism and accordingly has long been a subject of interest (SMITH et al., 2004). Previous studies have shown that measuring body size is an effective way to estimate the effects of exposure to environmental pollutants on wild animals. In mammals, reduced body size due to environmental stress has been extensively documented in the laboratory, but more seldom in the wild (PALMER & STROBECK, 1986; PANKAKOSKI et al., 1987; ALCÁNTARÁ & DIAZ, 1996; CAVALLINI, 1996; CRISTOFFER, 1991; NUNES et al., 2001a; RADWAN, 2003).

Additionally, many authors assume that animals that are heavier than predicted by their body size have more metabolizable tissue than individuals that are lighter than predicted by body size (DOBSON, 1992); it is unlikely that this extra mass is composed strictly of fat. Unless animals are depositing energy (fat) for a specific purpose such as migration or hibernation, it seems likely that the variation in condition reflects the variation in all constituents of body composition, including fat, protein, water and skeletal tissue (SCHULTE-HOSTEDDE, 2005).

An animal's body condition refers to its energetic state, so an animal in good condition has higher energy reserves (usually fat) than an animal in poor condition (SCHULTE-HOSTEDDE et al., 2001; SCHULTE-HOSTEDDE, 2005). In mammals, the amount of fat that an individual carries can have important fitness consequences (SCHULTE-HOSTEDDE et al., 2001). For instance, individuals with larger fat reserves may have better fasting endurance and a higher survival rate than individuals with smaller reserves (MILLAR & HICKLING, 1990). On the other hand, locomotion and predator avoidance can be compromised by heavy fat reserves (TROMBULAK, 1989). Still, measuring the body condition of live animals has been the subject of much recent debate (JAKOB et al., 1996; KOTIAHO, 1999; GREEN, 2001; HAYES & SNOKWILER, 2001; SCHULTE-HOSTEDDE et al., 2001; SPEAKMAN, 2001).

The study tested the hypothesis that mice from a polluted area 1) would exhibit a lower developmental stability (DS), 2) would have reduced body sizes and 3) would have an inferior body condition compared to mice from an unpolluted reference site. To test this hypothesis, developmental stability was assessed by determining the level of fluctuating asymmetry and the total amount of phenotypic variability. Body weight and length were also used as indices of body size, while an index of body condition presented the measure of body condition in *Apodemus agrarius.*

MATERIALS AND METHODS

Study areas

This study was performed at two areas in northern Serbia. The contaminated site (Pancevo) is the site of a large petrochemical and fuel storage complex. It is located approximately 15km northeast of the capital (Beograd) and includes an ammonia plant (founded in 1962), a factory for chemical fertilizers (founded in 1975), and a crude oil refinery (founded in 1968). Here it is necessary

to indicate certain chemical compounds, their metabolites and unwanted by-products, because they represent some of the most dangerous chemical pollutants producing long-term negative effects on the environment, human health and living organisms. About 700 tons of liquid ammonia (NH_3) and 500 tons of urea (CH_4N_2O) are processed daily, and about $8x10^3$ tons of hydrochloric acid (HCl, 33%), 109,600 tons of vinyl chloride monomer (VCM, C_2H_3Cl), $200x10^3$ tons of ethylene dichloride ($C_2H_4Cl_{12}$), $4850x10^3$ tons of crude oil products are produced annually. At the petrochemical plant, chlorinated solvents such as trichloromethane, tetrachloromethane, trichloroethane, dichloroethene, trichloroethene, and others closely associated with the unwanted by-products of PVC (polyvinyl chloride) production, were found in both soil and groundwater samples (GOPAL & DELLER, 2002). Additionally, VCM is a human carcinogen known to cause liver and blood tumors. VCM released into the atmosphere is protected from polymerization and remains dangerous for a long period for the environment and to humans. The combustion of VCM generates cyclic dioxins that are internationally recognized cancer inducers. Soot is extremely carcinogenic; its toxicity is attributable to high concentrations of polycyclic aromatic hydrocarbons; they are metabolically transformed by aquatic and terrestrial organisms are into carcinogenic and mutagenic metabolites.

The reference site (Cer), a forested area, is located 150km to the west of the polluted site, far from any known contamination.

Sampling and statistical treatment

From 1994-2000 (except in 1999), mice were trapped live using Longworth traps baited with mixture of sardines and grain. One hundred traps were set in the morning at intervals of approximately 5m, along 500m transects. The animals were collected the following morning.

In the laboratory, the mice were sacrificed and analyzed within three days after capture. All analyses (developmental stability, body size and body condition) were performed using the same individuals.

A total of 156 *Apodemus agrarius* were studied: 68 from the polluted area (34 males and 34 females; 24 juveniles and 44 adults) and 88 from the reference site (60 males and 28 females; 27 juveniles and 61 adults), which were the same used by VELICKOVIC (2004).

Statistical treatment for developmental stability indices

Developmental stability was assessed using quantitative characters presented as a number of paired foramina in the skull. Foramina, the small openings for nerves and blood vessels, were counted macroscopically under a binuclear microscope (16x magnification) on the right and left sides of the cleaned skull without a knowledge of where the animal had been trapped; the skulls were cleaned in a dermestid beetle (*Dermestes maculatus*) colony.

The abbreviations for foramina are (Fig. 1): *foramen parietalis* (F_1), *foramen dentale* (F_2), *foramen palatinum* (F_3), *foramen alveolaris maxillaris* (F_4), *foramen mentale* (F_5), *foramen angularis* (F_6).

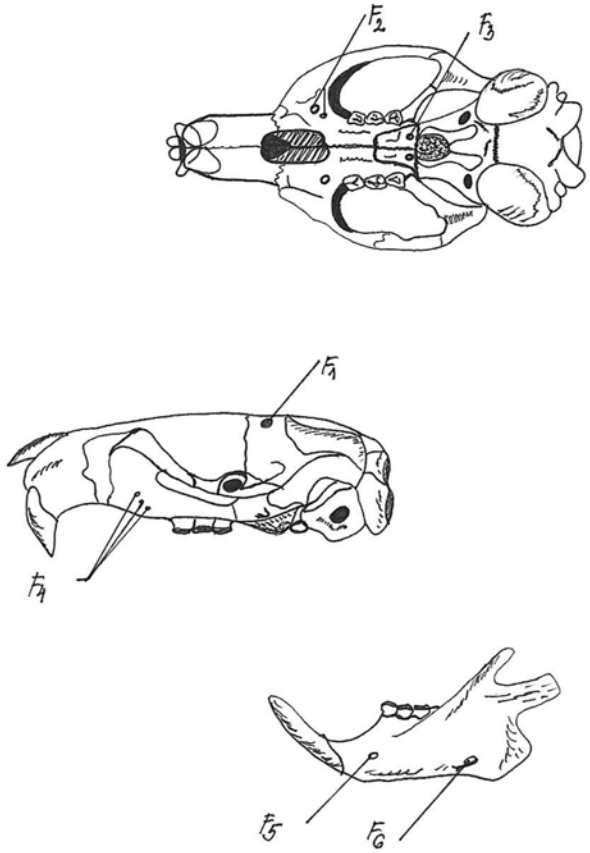

Fig. 1. – Skull foramina used in the study. See the text for abbreviation references.

This set of meristic (non-metric) characters was selected because it has been shown that meristic characters are amenable to analysis using quantitative genetic methods (FALCONER, 1989) and because developmental stability (measured as FA) has been assessed using the numbers of paired foramina in the skull (BERRY, 1969; ZAKHAROV & YABLOKOV, 1990; PANKAKOSKI et al., 1992; PERTOLDI et al., 1997).

Two indices were calculated separately for each character to describe developmental stability: (1) FA_4: index 4 of PALMER & STROBECK (1986), PALMER (1994) and (2) the total phenotypic variability of foramen numbers (PV), ZAKHAROV (1984).

(1) $FA_{4\ (foramina)} = Var\ (R_i - L_i)$

(2) $PV_{\ (foramina)} = Var\ (r_i + l_i)$,

where $(R_i - L_i)$ = signed asymmetry, R_i and r_i = value of the character on the right side of the skull, L_i and l_i = value of the character on the left side of the skull, Var = variance.

Before proceeding with the asymmetry analysis, statistical tests were carried out to detect the features confounding the analyses of FA (PALMER & STROBECK, 1986; PALMER, 1994) such as: directional asymmetry (DA) and antisymmetry (AS).

Firstly, the deviation from the normality of the (R_i -L_i) distributions was assessed using the Kolmogorov-Smirnov test of normality. These distributions were tested for significant skewness (g_1) and kurtosis (g_2) according to SOKAL & ROHLF (1981). Secondly, a one-sample *t*-test for a departure of the mean of (R_i - L_i) from an expected mean of zero was performed. Thirdly, for each character within samples a simple linear regression of absolute asymmetry $|(R_i - L_i)|$ on ($R_i + L_i$)/2 was assessed.

Because the FA and PV values are variances, statistical comparisons between samples can be detected by comparing the heterogeneity of variances. Although this was implemented by performing the *F*-test, a sequential Bonferonni correction (RICE, 1989) was applied to avoid "false" significant results. The Friedman test was used to compare the medians of variances.

Statistical treatment for body size and body condition measures

For each mouse, the measurements of body size were recorded as body length (BL) calculated by subtracting the tail length from the total length (1mm accuracy) and body weight (BW) measured to the nearest 0.1mg (embryo weights were deducted from the body weight of pregnant females). The expected weight for a given length was obtained from a linear equation of the logarithms of BW and BL (in males and females, using an analysis of covariance for slopes). Subsequently, an index of body condition (BCI) was calculated for each mouse as the quotient between observed and expected values of BW, (NUNES et al., 2001a).

All these variables were checked for normal distribution using the Kolmogorov-Smirnov test of normality.

The analyses of the main effects of site, sex, age category and all possible interactions of these variables on BW, BL and BCI were performed using three-way ANOVA (analysis of variance) procedures.

RESULTS

Preliminary results of asymmetry analyses

For the three (R_i-L_i) distributions considered in this study, normality was rejected: (in Cer: *foramen mentale* d=0.374, P<0.01; in Pancevo: *foramen mentale* d=0.371, P<0.01 and *foramen angularis* d=0.165, P<0.05).

The results of the regression analyses between $|R_i$-$L_i|$ and (R_i+L_i)/2 were significant: at Cer (for *foramen alveolaris maxillaris*, r=0.400, P=0.000; for *foramen mentale*, r=0.747, P=0.000 and for *foramen angularis*, r=0.260, P=0.016) and at the Pancevo site (for *foramen parietalis* r=0.830, P=0.000; for *foramen dentale* r=0.677, P=0.000; for *foramen palatinum* r=0.273, P=0.027; for *foramen alveolaris maxillaris* r=0.399, P=0.001, and for *foramen mentale* r=0.628, P=0.000).

For cases of significant linear regression of absolute asymmetry $|(R_i - L_i)|$ on ($R_i + L_i$)/2 value, the index chosen to measure FA (FA_4) was recalculated as the value of (R_i-L_i) divided by (R_i+L_i)/2, index 6 (FA_6 of PALMER & STROBECK, 1986; PALMER, 1994)

$$FA_{6\ (foramina)} = Var\ [(R_i-L_i)/\ ((R_i+L_i)/2)]$$

where (R_i - L_i) = signed asymmetry, R_i = value of the character on the right side of the skull, L_i = value of the character on the left side of the skull, Var = variance.

To ensure the corrections' successful implementation, I again correlated the corrected (R_i-L_i) values [(R_i-L_i)/ (R_i+L_i)] with ((R_i+L_i)/2).

The results of the *t*-test showed that there were no significant departure of the mean of (R_i-L_i) from the expected mean of zero in both samples (no DA asymmetry is present, all P>0.05). Three out of twelve (R_i-L_i) distributions were significantly skewed (two in Cer: for *foramen parietalis*, g_1=-35.671, P<0.01, and for *foramen angularis* g_1=2.145, P<0.05, and one in Pancevo: for *foramen parietalis* g_1=12.445, P<0.01). A significant platikurtic or bimodal distribution (-g_2 value) was not found; thus no antisymmetry is present.

Developmental stability indices

Higher values of FA were found in the polluted area for all characters except for *foramen alveolaris maxillaris*. The *F*-test results also showed that in five out of six characters the variances describing FA differ significantly between the two areas (P_r=0.05). Specifically, for four out of these five characters (*foramen parietalis*, *foramen dentale*, *foramen palatinum*, and *foramen angularis*), FAs were higher (developmentally less stable) in the contaminated area than in the reference area. For *foramen mentale* however, FA was significantly higher in the control area, Table 1.

TABLE 1

Comparison of fluctuating asymmetry (Asymmetry) and total phenotypic variability (Variability) in foramen numbers between the polluted (Pancevo=$P_{AN.}$) and control (Cer=C) areas.

	Asymmetry			Variability		
Foramen	C (N)	$P_{AN.}$ (N)	P_r	C (N)	$P_{AN.}$ (N)	P-value
F_1	0.112 (86)	0.225 (67)	*	0.012 (87)	0.371 (67)	**
F_2	0.558 (85)	1.108 (68)	*	0.856 (87)	1.340 (68)	**
F_3	0.304 (85)	0.623 (66)	*	38.854 (86)	53.421 (66)	NS
F_4	0.459 (86)	0.286 (67)	*	16.001 (86)	17.865 (67)	NS
F_5	0.128 (86)	0.125 (68)	NS	0.304 (86)	0.362 (68)	NS
F_6	0.263 (86)	4.682 (68)	*	16.327 (86)	16.355 (68)	NS

N =sample size, NS =statistically not significant, Pr =revised probability values (results of the sequental Bonferonni correction), * = significant at P<0.05, ** = significant at P<0.01

Moreover, the results obtained results demonstrated significant differences in FA levels between sites within the same sex (males from the polluted area had a significantly higher FA value for *foramen parietalis*; F=5.547, P=0.025).

Concerning age categories, a statistically significant difference in FA levels was observed between juvenile and adult mice from the polluted area. Juvenile animals had significantly higher FAs for two characters ($F_{foramen\ dentale\ (23,26)}$ =9.313, P<0.05, and $F_{foramen\ palatinum\ (23,26)}$ =7.653, P<0.05) compared to adults from the same site.

However, when all the foramina were considered together, a three-way analysis of variance revealed that there was no significant interaction between the factors (sex x age category x site) on the FA (average value), Table 2. The average FA value in foramen numbers was calculated by dividing the sum of FAs by the total number of characters analyzed.

TABLE 2

Results of the three-way analysis of variance relating the FA (average value) with age category, sex and site.

Source of variation	df	MS	F-ratio	P-value
Age category	1	0.059	0.114	0.736 NS
Sex	1	0.004	0.008	0.930 NS
Site	1	0.586	1.121	0.291 NS
Age category x Sex	1	0.688	1.316	0.253 NS
Age category x Site	1	0.037	0.070	0.791 NS
Sex x Site	1	0.987	1.887	0.172 NS
Age category x Sex x Site	1	0.007	0.014	0.908 NS
Error	137	0.523		

NS = statistically not significant

PV values differ at the investigated sites; a comparison of PVs indicated higher values in the polluted area for all analyzed characters. For two out of six characters (*foramen parietalis* and *foramen dentale*), the PV values were significantly higher in the polluted area than in the reference area, Table 1.

When sexes were separated and tested for PV, the results indicated a difference between the sites within sexes. Within both sexes, the PVs were significantly higher in mice from the polluted area compared with mice from the unpolluted site (for males: $F_{foramen\ parietalis}$ =7.354, P=0.008, and for females: $F_{foramen\ dentale}$ =4.524, P=0.038).

When all the foramina were considered together, the FA does not differ significantly between the two areas, although the PV was significantly higher at the contaminated site (Friedman's test results for median values, P<0.014), Fig. 2.

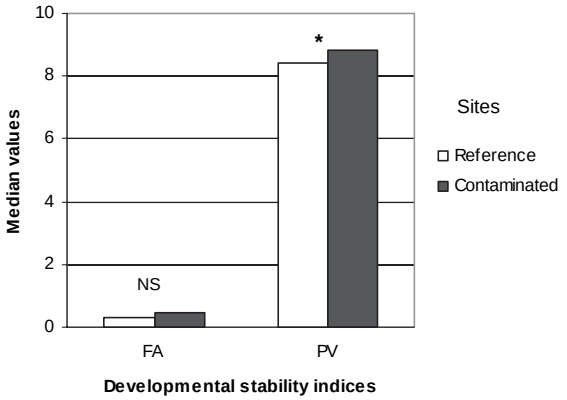

Fig. 2. – Comparison of developmental stability indices (fluctuating asymmetry = FA and total phenotypic variability = PV) in *A. agrarius* in the polluted and control areas. The values on the y-axis are medians (FA; PV), NS= statistically not significant, * = statistically significant at P<0.014.

Body size and body condition

Body length, body weight and an index of body condition produced normal distributions at both sampling sites. However when each sex was analyzed separately, normality was rejected for the females from the control area (for body length, P<0.05) and for males from the polluted area (for body weight, P<0.05).

Results also indicated that (1) the coefficients of variation (CVs) for body size measures were lower in mice from the polluted area compared to mice from the reference site except for BL and (2) CVs were largest for body weight and the smallest for body length within the sites, Table 3.

When CVs were computed by age category, site and sex, the highest value was detected for the index of body condition in adult males from the reference site (CV=19.719), while the smallest value exists for body length in juvenile males from the polluted area (CV=3.351).

TABLE 3

Descriptive statistics for body measurements in Cer (reference) and Pancevo (contaminated) sites

Site	Variable	N	Mean ± S.D.	Min.	Max.	CV
Cer	BW (g)	88	18.717±4.420	11.100	32.703	23.605
	BL (mm)	89	95.521±6.998	82.000	116.000	7.322
	BCI	88	6.401±1.212	4.082	10.189	18.910
Pancevo	BW (g)	75	17.933±3.725	11.000	30.500	20.800
	BL (mm)	75	92.346±7.836	62.000	116.000	8.494
	BCI	75	6.228±1.030	4.167	9.297	16.530

N = sample size, Mean = mean value, S.D. = standard deviation, Min. = minimum, Max. = maximum, CV = coefficient of variation, BW = body weight, BL= body length, BCI = index of body condition

Linear equations of the logarithms of BW and BL were obtained for each sex separately: for males (y=2.083x–6.555, r^2=0.730, P=0.000) and for females (y=1.597x–4.369, r^2=0.709, P=0.000). The models obtained were highly significant for both sexes (P=0.000). However their expected power is low: 30.1% and 26.8% of the variance explained for males and females, respectively (Fig. 3a and Fig. 3b).

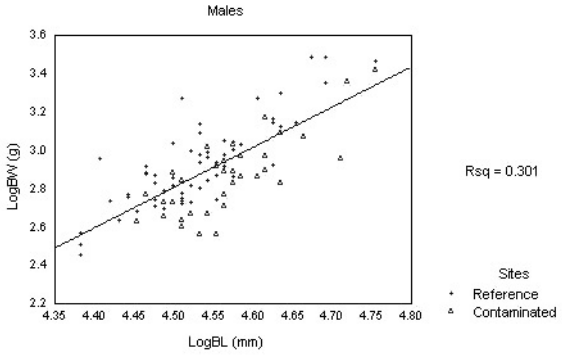

Fig. 3a. – Relationship between body weight (BW) and body length (BL) for male *A. agrarius* specimens.

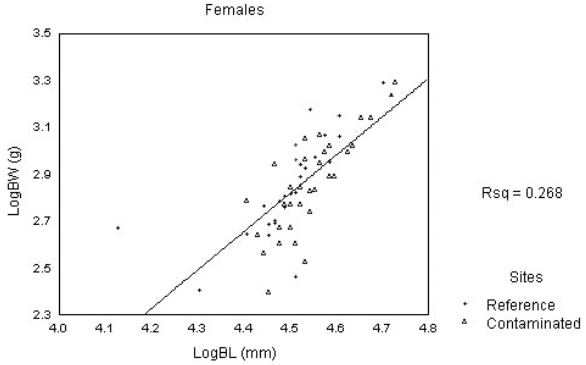

Fig. 3b. – Relationship between body weight (BW) and body length (BL) for female *A. agrarius* specimens.

The effects of sex, age category, site and interactions of these variables on BW, BL and BCI were assessed by using the ANOVAs (Table 4). No effects of possible interactions of sex, age category and site on BW, BL and BCI values were observed. Significant differences were however detected in BW between age categories: (19.665$_{(g)}$ vs. 15.574 $_{(g)}$, for adults and juvenile individuals, respectively; F= 45.686, P=0.000).

In BL, significant differences were observed between sexes: (94.623 $_{(mm)}$ vs. 92.677 $_{(mm)}$, for males and females respectively; F=4.465, P=0.040), between age categories (96.286 $_{(mm)}$ vs. 88.717 $_{(mm)}$, for adults and juvenile individuals, respectively; F=48.268, P=0.000) and between sites (95.520 $_{(mm)}$ vs. 92.348 $_{(mm)}$, in the control area and the polluted area, respectively; F=16.770, P=0.000).

BCI in adults was significantly higher than in juveniles (6.663 vs. 5.589; F=38.783, P=0.000) and in mice from the reference site than in the polluted area (6.403 vs. 6.234; F=5.757, P=0.020).

TABLE 4

Table 4. Results of the three-way ANOVAs relating BW (body weight), BL (body length) and index of body condition (BCI) with sex, age category, site and all their possible interactions

Variable	Effects of interactions	F - ratio	P - value
BW	Sex (1)	0.099	0.753
	Age category (2)	45.694	0.000*
	Site (3)	1.084	0.299
	1x2	0.014	0.905
	1x3	2.547	0.112
	2x3	0.070	0.791
	1x2x3	0.081	0.775
BL	Sex (1)	4.471	0.036*
	Age category (2)	48.270	0.000*
	Site (3)	16.772	0.000*
	1x2	0.495	0.482
	1x3	0.851	0.357
	2x3	2.933	0.088
	1x2x3	0.059	0.807
BCI	Sex (1)	0.054	0.815
	Age category (2)	38.781	0.000*
	Site (3)	5.761	0.017*
	1x2	0.132	0.716
	1x3	2.481	0.117
	2x3	0.796	0.373
	1x2x3	0.207	0.650

* = statistically significant at P<0.05

Finally, to investigate the relative importance of the three potential biomarkers, a multivariate analysis of variance (MANOVA procedure) was performed. The MANOVA's results revealed the significant main effects of age category and site on FA (average value), size and body condition, Table 5. The MANOVA's results also showed significant interaction for sex x site. Significant test results for the MANOVA procedure were based on *F* statistics derived from Wilks' lambda.

TABLE 5

MANOVA`s results for the effects of sex, age category, site and their interaction on FA, body size and body condition values in *Apodemus agrarius*.

Source of variation	$df_{(1,2)}$	Wilks`s lambda	P - value
Sex	3,141	0.974	0.332
Age category	3,141	0.728	1.84×10^{-9}***
Site	3,141	0.738	4.97×10^{-9}***
Sex x Age category	3,141	0.990	0.489
Sex x Site	3,141	0.950	0.028*
Age category x Site	3,141	0.982	0.277
Sex x Age category x Site	3,141	0.996	0.742

*=P<0.05; ***=P<0.001

DISCUSSION

Assessing the impacts of environmentally-generated stress factors on natural plant and animal populations is a complex task because organisms in the environment are

exposed to a diverse range of uncontrolled variables such as weather conditions, parasites, mixtures of pollutants). Within the environment, the synergistic and antagonistic effects of these factors are not always fully understood. Additionally, the presence of environmental pollutants in small mammals is relatively easy to analyze, but it is difficult to measure their effects on population parameters. In fact, VALENTINE et al. (1973) and PARSONS (1992) concurred that pollutants must reach substantial concentration levels before they observably affect population density, viability and breeding success. However, a positive benefit of *in situ* environmental quality monitoring studies is that the obtained results can tell us what really happens in the environment. The experimental design employed in this study is unique because only a few studies have been conducted with *A. agrarius* and because no investigator has previously used this combination of endpoints in an environmental quality monitoring study.

Several researchers have examined the variability in the numbers of paired foramina as an expression of developmental stability (measured as FA) and have found differences between natural populations (BERRY, 1969; ZAKHAROV & YABLOKOV, 1990; PANKAKOSKI et al., 1992). Specifically, these authors found that a disruption in developmental stability was observed in animals affected by different types of environmental pollutants (but see SCHANDORFF, 1997).

The results presented here demonstrated that the level of FA in the polluted area was increased for all foramina except for *foramen alveolaris maxillaries*, and the developmental stability of the same characters decreased.

The fact that FA levels were significantly different between sites within the same sex indicates that it is possible that certain pollutants affect sexes differentially. The study also indicated that the progeny of animals with long-term exposure to environmental pollutants had significantly higher levels of FA for *foramen dentale* and *foramen palatinum*. Thus, the increased FA in mammals seems to be a consequence of stress experienced by mothers and fetuses during pregnancy or lactation (SIEGEL & DOYLE, 1975b) and that the developmental disturbances occurred during the prenatal life or at the early stages of development. On the other hand, the use of energy for stress tolerance that could otherwise be used for developmental control, growth, reproduction, and survival (HOFFMAN & PARSONS, 1989; PARSONS, 1990; ALEKSEEVA et al., 1992; OZERNYUK et al., 1992) impose a distinct developmental stress.

Interestingly, in a 3-way ANOVA correcting for age and/or sex, no effect of site on average FA is found. One of possible explanations would be that there is no replication, and thus by definition the individuals within the two sampling sites are no real replicates.

It is worth remarking that the skulls of rodents have been frequently employed in FA studies; the lower jaw of a mouse has long served as a model system for studying the development and evolution of complex morphological structures (KLINGENBERG, et al., 2003). The measurements that are classically recorded for the mandible (mandible mass, mandible size, maximum width and length of the lower molars, the height and length of the mandible) indicated that increased FA levels in the lower jaw (and associated tooth) dimensions in mice responded to various kinds of stress (SIEGEL & DOYLE, 1975c; SIEGEL et al., 1977; LEAMY, 1984; ATCHLEY et al., 1984; PANKAKOSKI et al., 1992; GILEVA & NOKHRIN, 2001; VELICKOVIC, 2004). However for all of these studies suggesting that stress can increase FA, there are others where no effects, or even the opposite effects, of stress on FA have been observed (LEAMY et al., 1999a; MARKOW, 1995; WOODS et al., 1999). Thus, the results obtained in my studies and in other investigations corroborate the potential use of FA on both metrical and meristic characters, as a good diagnostic procedure for assessing stress tolerance. Concurring with this view, LEAMY & KLINGENBERG (2005) suggest that FA may play some kind of role as a fitness indicator.

The data presented here also indicates that both sexes had significantly higher PVs in the polluted area. Interestingly, when all foramina were considered together, the PV value was significantly higher in the polluted area than at the control site. This finding is consistent with similar results in an earlier study (PANKAKOSKI et al., 1992), whose authors revealed that the total phenotypic variance is greater in the common shrew (*Sorex araneus*, Linnaeus 1758) associated with heavy metal pollution than in the reference areas.

The present study therefore indicates that developmental stability is one of the most general characteristics in individual developmental processes and is also a sensitive parameter that can be used for the biomonitoring of natural populations' conditions (SOULÉ, 1967; JONES, 1987; ZAKHAROV, 1981; 1987). An analysis of developmental stability may therefore potentially reveal changes in a population's condition, the changes not yet reflected by disturbances of individual viability (YABLOKOV, 1986). Moreover, by studying developmental stability it might also be possible to assess the synergetic effects of toxic compounds or interactions between pollutants and other stressful factors (ZAKHAROV et al., 1991), which are either complex or impossible to study by other means.

Certain data from the body size and body condition analyses found in this study are similar to findings reported by NUNES et al. (2001a) for the Algerian mouse (*Mus spretus*, Lataste 1883) inhabiting an area contaminated with heavy metals. However, the data from *M. spretus* has been used more as a reference than for direct comparisons. Mice (from both studies) trapped at the contaminated sites have reduced BW as well as BL values. In NUNES et al. (2001a), all analyzed *Mus spretus*, except non-reproductive males, weighed less at the metal-polluted site compared with mice inhabiting the unpolluted area. The reduction of bone lengths and tooth dimensions in laboratory rats raised under effects of various stresses, such as extreme temperatures or noise, has been demonstrated by DOYLE et al. (1977). On the other hand, lead poisoning in laboratory rats results in decreased body weight (GOYER et al., 1970). Thus, the small sizes of mice found in contaminated areas may be explained by the negative effects of chemical pollutants from the environment. However, other causes, such as nutrition quality/availability, cannot be ruled out.

M. spretus and *A. agrarius* mice from the reference areas exhibited better body condition than mice from the

polluted sites, revealed by the significantly higher BCI value. Furthermore, the same trend was observed for adults from the investigated localities; compared with juveniles, the adults exhibited better body condition than the juveniles. This observation can be explained as a consequence of normal ontogenetic development; because juveniles reach adult length before they reach adult mass, they will normally go through a period in which they are long relative to their mass. Another possibility may result from the deposition of fat reserves during the breeding season (MILLAR, 1975).

The MANOVA's results revealed the significant effects of age category, site and interaction for sex x site on FA (average value), size and body condition.

Conclusions and prospects for future studies

The present study indicated that developmental stability indices, combined with body size measures as well as an index of body condition can be used to detect, analyze and evaluate the effects of environmentally-generated stress factors on natural populations of small mammals. Therefore, I propose the use of this combination of endpoints as an ecotoxicological biomarker in pollution monitoring.

In conclusion, the results obtained from previous researches and the present study demonstrated that despite *A. agrarius's* high tolerance to contaminants (PETROV, 1992), it may be an important species for environmental quality monitoring.

For future work, it would be interesting to compare the results of investigations performed on *Apodemus agrarius* with new data obtained from other species of the genus *Apodemus* and/or other species of small mammals indigenous to Serbia.

However, it would be important to consider the following: 1) to increase number of investigated localities in order to avoid pseudoreplication. According to HURLBERT (1984) pseudoreplication is defined as the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates are not statistically independent 2) that each polluted site should be scored for quantitative variables such as types and concentrations of environmental pollutants 3) a larger sample size is necessary because increases statistical power (COHEN, 1988; YEZERINAC et al., 1992).

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Évaluation de l'effet d'un système de refuge sur la survie et la croissance des alevins de *Heterobranchus longifilis* élevés en cage flottante

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RÉSUMÉ. La présente étude a consisté à évaluer en cage flottante (1m^3) l'effet de l'introduction d'un système de refuge (bandelettes plastiques) sur la survie et la croissance des alevins de *Heterobranchus longifilis*. Trois séries d'expériences ont été menées. La première [poids moyen initial (P_{mi}): 0.9±0.1g ; densité: 100 individus/m^3] a porté sur l'évaluation de l'effet de la coloration (blanche, bleue, noire) du système de refuge sur la survie et la croissance. Après 90 jours d'élevage, aucune influence (p>0.05) de la coloration des bandelettes qui constituent le système de refuge n'a été observé sur le taux de survie (Ts) et le poids moyen final (Pmf). Par contre, comparativement aux cages sans système de refuge, ces paramètres ont été nettement améliorés (p<0.05) avec l'utilisation du dispositif de refuge (respectivement 70.9-74.0% et 16.2-17.1g contre 64% et 11.8g pour les cages sans refuge). La deuxième expérience (durée: 90 jours ; Pmi: 0.9±0.1g) a permis de suivre, pour les deux types de cages testées, l'effet de quatre densités de mise en charge (100, 200, 500 et 1000 individus/m^3) sur la survie et la croissance. D'une manière globale, le système de refuge a produit les meilleurs résultats. La densité de 100 individus/m^3 a donné les meilleurs taux de croissance et de survie. La troisième expérience (durée: 60 jours ; densité: 100 individus/m^3) a porté sur la détermination de l'effet du système de refuge et du poids moyen initial (13.2±1.3g, 25.2±2.5g, 40.3±4.0g et 60.3±6.0g) sur la survie et la croissance dans les deux types de cages testés. Les résultats de cette expérience ont permis de mettre en évidence deux types d'effets du système de refuge: (1) un effet positif chez les poissons de poids compris entre 13.2 et 29.2g et (2) un effet négatif chez les poissons de poids compris entre 60.3 et 92.0g.

MOTS CLÉS : *Heterobranchus longifilis*, système de refuge, survie, croissance

Evaluation of a man-made shelter's effects on survival and growth of *Heterobranchus longifilis* fry under cage culture

ABSTRACT. The effects of shelter made from inert synthetic shade materials on the survival and growth of *Heterobranchus longifilis* fingerlings were evaluated under cage (1m^3) culture conditions in man-made Lake Ayame (Côte d'Ivoire) from September 2003 to March 2004 (180 days). In the first experiment, fishes (initial mean weight: 0.9±0.1g ; density: 100 fishes/m^3) were reared during 90 days in either unsheltered or sheltered (white, blue or black synthetic shade material) cages. Survival and final mean weight were not affected by the colour of the shelter but were significantly higher (p<0.05) when shelter was provided (70.9-74.0% and 16.2-17.1g for sheltered cages *vs* 64.0% and 11.8g for unsheltered). During the second experiment (90 days), *H. longifilis* (initial mean weight 0.9±0.1g) were reared at four different stocking densities (100, 200, 500 and 1000 fishes/m^3) in both sheltered and unsheltered cages. Survival and final mean weight noted were better in the sheltered cages. In both sheltered and unsheltered cages, these parameters were better for cages of 100 fishes/m^3 and worst for cages of 1000 fishes/m^3. In the third experiment (stocking density: 100 fishes/m^3), *H. longifilis* of four different initial mean weight (13.2±1.3g, 25.2±2.5g, 40.3±4.0g and 60.3±6.0g) were reared during 60 days in either unsheltered or sheltered cages. Results showed two different effects of the shelter on survival and final mean weight of fishes: (1) positive effect for fishes weighting from 13.2 to 29.2g, and (2) negative effect for fishes weighting from 60.3 to 92.0g.

KEY WORDS : *Heterobranchus longifilis*, man-made shelter, survival, growth

INTRODUCTION

Le poisson-chat africain *Heterobranchus longifilis* Valenciennes, 1840, utilisé en pisciculture en Afrique de l'Ouest, est une espèce chez laquelle le cannibalisme est très développé aux stades larvaire et juvénile (Otémé & Gilles, 1994 ; Otémé et al., 1996). Ce phénomène débute quatre jours après l'éclosion des larves et devient insignifiant à partir d'un poids moyen de 30g (Baras et al., 1999). De ce fait, les stades larvaire et juvénile sont considérés comme les plus délicats de l'élevage de ce poisson, étant donné les taux élevés de mortalité liés, entre

autres, au phénomène de cannibalisme (Otémé & Gilles, 1994; Otémé et al., 1997; Gilles et al., 2001).

Cependant, chez certaines espèces cannibales, quelques travaux ont permis de mettre au point des techniques permettant d'améliorer le taux de survie lors de l'élevage des larves et des juvéniles: (1) l'utilisation d'alevins de taille homogène pour le démarrage des cultures (Otémé & Gilles, 1994; Otémé et al., 1996), (2) la réalisation de tris réguliers au cours de l'élevage (Gilles et al., 2001; Baras et al., 2000), (3) l'utilisation d'un aliment à haute teneur en protéine (Gilles et al., 2001), (4) la réalisation de l'élevage dans des conditions optimales de température de manière à raccourcir le temps de développement durant lequel l'exercice du cannibalisme est accru (Yada & Furukawa, 1999), (5) la réalisation de l'élevage à l'obscurité (Hossain et al., 1998; Appelbaum & Kamler, 2000) et (6) la réalisation de l'élevage à des densités optimales de mise en charge (Haylor, 1991; Hengsawat et al., 1997). Bien que de nombreuses méthodes aient été testées pour réduire la mortalité due au cannibalisme, aucune d'entre elles n'a permis à ce jour de la maintenir à des seuils acceptables. Très peu d'expériences basées sur l'introduction dans le milieu d'élevage de dispositifs pouvant servir de refuges pour les larves ou alevins les plus faibles ont été menées. En milieu naturel, *Heterobranchus longifilis* colonise préférentiellement les biotopes difficiles d'accès (herbiers de bordure) (Legendre, 1991). Partant de ces observations, l'hypothèse de travail de la présente étude a consisté à vérifier si l'introduction dans le milieu d'élevage d'un nuage de bandelettes plastiques (polyéthylène) enchevêtrées peut servir de refuges pour des larves ou alevins de *H. longifilis*.

La présente étude est entièrement réalisée en cage flottante. Compte tenue du fait que les poissons sont sensibles à la coloration de leur enceinte d'élevage (Fontaine & Le Bail, 2004), elle se propose de suivre dans un premier temps, l'effet d'un système de refuge constitué de bandelettes plastiques de différentes couleurs sur la survie et la croissance des alevins de *H. longifilis*. Dans un second temps, elle déterminera l'effet de la variation de la densité de mise en charge et du poids moyen initial sur la survie et la croissance des alevins de *H. longifilis*.

MATÉRIEL ET MÉTHODES

Cette étude a été réalisée entre septembre 2003 et mars 2004 (180 jours) dans le lac de barrage d'Ayamé (Côte d'Ivoire). Les cages flottantes utilisées dérivent de celles mises en œuvre par Coche (1978) et Cavailles et al. (1981). D'un volume utile de $1m^3$, chaque cage est constituée d'une armature en bois ($1 \times 1 \times 1.5$m) habillée de grillage plastique de type NORTENE de 1mm (expérience 1 et 2) ou 10mm (expérience 3) de vide de maille.

Le système de refuge utilisé est constitué de bandelettes plastiques (polyéthylène) enchevêtrées (Fig. 1). Ces bandelettes (0.5 à 1m de longueur, 3mm de largeur et 0.2mm d'épaisseur) sont localement disponibles en couleur blanche, bleue et noire. Elles sont utilisées en industrie pour la confection de sacs couramment utilisés pour la conservation des produits agricoles. Des cages avec système de refuge (ou cages aménagées) ont ainsi été mises au point en fixant sur le fond de chacune d'elles 2kg de bandelettes (environ 25% du volume utile de la cage).

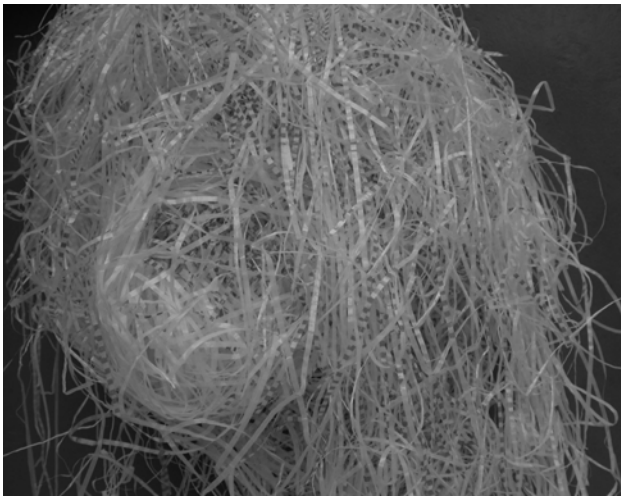

Fig. 1. – Bandelettes plastiques (polyéthylène) utilisées comme système de refuge.

Les alevins utilisés au cours de ce travail ont été obtenus en écloserie par fécondation artificielle (Slembrouck & Legendre, 1988) à partir de géniteurs capturés dans la rivière Bia (Côte d'Ivoire).

Trois séries d'expériences ont été effectuées. L'expérience 1, réalisée à la densité de mise en charge de 100 individus/m^3, a consisté à suivre sur une durée de 90 jours, l'effet du système de refuge et de sa coloration sur la survie et la croissance des alevins de poids moyen initial égal à 0.9±0.1g. L'expérience 2 (durée: 90 jours; Pmi: 0.9±0.1g) a permis de tester l'effet de quatre densités de mise en charge (100, 200, 500 et 1000 individus/m^3) sur la survie et la croissance des alevins (poids moyen initial 0.9g) en cages aménagées et en cages non aménagées (cages sans système de refuge). L'expérience 3 (durée: 60 jours; densité de mise en charge: 100 individus/m^3) a permis de faire une étude comparative de la survie et de la croissance entre les cages aménagées et les cages non aménagées pour des poissons de différents poids moyens initiaux notamment 13.2±1.3g, 25.2±2.5g, 40.3±4.0g et 60.3±6.0g. Dans toutes ces expériences, les cages témoins (ou cages non aménagées) ne contiennent pas de système de refuge. Chaque traitement a été répliqué trois fois, soit 12 cages pour l'expérience 1 et 24 cages pour chacune des expériences 2 et 3. Les poissons ont été nourris tous les jours (à l'exception des jours de pêche de contrôle) à 6h30, 12h30 et 18h30 avec un aliment commercial (Aliment FACI) titrant 35% de protéines brutes. La composition de cet aliment est présentée dans le Tableau 1. Pour les expériences 1 et 2, l'aliment a été distribué aux alevins sous forme de farine les 30 premiers jours et ensuite sous forme de granulés de 2mm de diamètre les 60 jours suivants. Au cours de l'expérience 3, ces granulés de 2mm de diamètre ont été distribués pendant toute la durée de l'essai. Le taux de rationnement journalier était de 10% (expériences 1 et 2) et de 5% (expérience 3) du poids vif selon les recommandations de Hem et al. (1994) et Otémé

et al. (1996; 1997). Ces différents taux ont été réajustés tous les 15 jours après une pêche de contrôle. Tous les jours, les alevins morts flottant à la surface de l'eau ont été comptés et examinés individuellement. Ont été considérés comme victimes d'une mortalité naturelle, les individus morts dont le corps ne présentait aucune trace de lésion. Quant aux poissons morts dont le corps était incomplet ou lésé (cannibalisme de type I) et ceux ayant disparu (cannibalisme de type II), ils ont été considérés comme victimes de cannibalisme (HECHT & APPELBAUM, 1988; BARAS et al., 1999).

TABLE 1

Composition de l'aliment commercial selon le fabricant FACI (Fabrication d'Aliments Composés Ivoiriens: 18 BP 686 Abidjan 18, Côte d'Ivoire).

Ingrédients	Proportions
Farine de poisson	41%
Issues de céréales (son de blé et de riz)	35%
Tourteaux de coton et de soja	13%
Tourteaux de coprah	10%
Prémix vitamines et minéraux	1%
Caractéristiques analytiques	
Protéines brutes	35%
Matière grasse brute	6%
Matière minérale	10%
Matière cellulosique brute	5%
Calcium	2,3%
Phosphore	1%
Sodium	0,4%
Vitamine C	400mg/kg
Vitamine A	10 000U.I./kg
Vitamine D3	3 000U.I./kg
Vitamine E	135mg/kg

Les caractéristiques physico-chimiques du milieu d'élevage ont été mesurées 3 fois par semaine à l'aide d'un oxymètre de modèle WTW OXY 330 (pour la température et le taux d'oxygène dissous), d'un pH-mètre de modèle WTW pH 330 (pour le pH) et d'un disque de Secchi de diamètre 30cm (pour la transparence). Les caractéristiques physico-chimiques mesurées tout au long des trois expériences sont consignées dans le Tableau 2. Les valeurs enregistrées sont comprises dans la fourchette admise en pisciculture (WESTERS, 1979; MCDONALD, 1983; DÉLINCÉ, 1992).

En fin d'expérience, le contenu de chaque cage a été entièrement compté et pesé (au g près) et au moins 30% de la population a été pesée individuellement (au 1/10g près). A partir des données récoltées, le taux de survie Ts (%), le taux de mortalité Tm (%), le taux de cannibalisme Tc (%), le poids moyen final Pmf (g), la croissance journalière Cj (g/j), le coefficient de variation du poids Cv (%) et le taux apparent de conversion alimentaire Taca ont été calculés selon les formules suivantes:

TABLE 2

Caractéristiques physico-chimiques enregistrées au cours de l'élevage de *Heterobranchus longifilis* dans le lac de barrage d'Ayamé de septembre 2003 à mars 2004 (Temp=Température; O_2=Taux d'oxygène dissous; Trp=Transparence; min=valeur minimale; max=valeur maximale).

		Temp (°C)	O_2 (mg/l)	pH	Trp (mm)
Expérience 1	Min	23	3.9	6.5	1008
	Max	31.5	6.9	7.8	1310
Expériences 2 & 3	Min	22	4	6.4	1000
	Max	32.1	6.7	7.7	1320

Ts (%)=nf/ni × 100, avec nf=nombre final de poissons et ni=nombre initial de poissons.

Tm (%)=nm/ni × 100, avec nm=nombre final de poissons retrouvés morts.

Tc (%)=nd/ni × 100, avec nd=nombre de poissons ayant disparus de la cage.

Pmf (g)=Pt/nf, avec Pt=poids total (g).

Cj (g/j)=(Pmf-Pmi)/t, avec Pmf=poids moyen final (g), Pmi=poids moyen initial (g), et t=durée de l'élevage en jours.

Cv (%)=100 × (écart type/poids moyen).

Taca=Pn/A, avec Pn: poids total sec de l'aliment distribué (g), A: biomasse produite (g)

Les résultats sont présentés sous forme de moyennes ± écarts types entre triplicats. Les comparaisons ont été réalisées en procédant à une analyse de variance (Anova, expérience 1) ou de covariance (Ancova, expérience 2). Lorsque ces tests révélaient une différence significative, des comparaisons "*Post Hoc*" (Least Significant Difference: LSD) ont été exécutées. Les moyennes des traitements de l'expérience 3 ont été comparées deux à deux à l'aide du test de Student. La relation entre la densité (expérience 2) et les paramètres de croissance et de survie a été étudiée à l'aide de l'analyse de la régression linéaire. Dans tous ces tests statistiques, les différences ont été considérées significatives au seuil de 5%. Les analyses ont été effectuées à l'aide du programme STATISTICA 6.0 (Statsoft, Inc.).

RÉSULTATS

Expérience 1

Étude de l'effet du système de refuge et de sa coloration sur la survie et la croissance des alevins de *Heterobranchus longifilis:*

Après 90 jours d'élevage, les valeurs les plus intéressantes du poids moyen final Pmf (17.1±2.4g: refuge noir), du taux de survie Ts (74.0±2.8%: refuge bleu), de la croissance journalière Cj (0.18±0.02g/j: refuge noir), du coefficient de variation du poids Cv (30.0±2.3%: refuge blanc), du taux de cannibalisme Tc (17.3±1.3%: refuge bleu), du taux de mortalité Tm (10.0±1.4%: refuge bleu) et du taux apparent de conversion alimentaire Taca (3.57±0.17: refuge noir) ont été observées au niveau des cages aménagées (Tableau 3). A l'exception du coefficient de variation du poids final et du taux apparent de conversion alimentaire (Anova, $p>0.05$) la différence entre ces deux systèmes est statistiquement (Anova, $p<0.05$) significative. Parmi les cages aménagées, aucun effet (Anova, $p>0.05$) de la coloration (blanc, bleu et noir) du système de refuge n'a été observé ni sur les paramètres de survie (Tc, Tm et Ts), ni sur les paramètres de croissance (Pmf, Cv, Cj et Taca).

TABLE 3

Paramètres de survie et de croissance de *Heterobranchus longifilis* après 90 jours d'élevage en cage flottante sans système de refuge et en cage flottante munie de système de refuge de trois colorations différentes (bandelette synthétique blanche, bleue et noire) dans le lac de barrage d'Ayamé (Pmi=poids moyen initial, Pmf=poids moyen final, Cv=coefficient de variation du poids, Tc=taux de cannibalisme, Tm=taux de mortalité, Ts=taux de survie, Cj=croissance journalière et Taca=taux apparent de conversion alimentaire). Pour chaque ligne, les valeurs portant des lettres différentes sont significativement différentes (*Post Hoc*: LSD, $p<0,05$); les écarts-types ont été calculés entre triplicats.

	Sans refuge	Refuge (blanc)	Refuge (bleu)	Refuge (noir)	*p*
Pmi (g)	0.9±0.1	0.9±0.1	0.9±0.1	0.9±0.1	>0.05
Pmf (g)	11.8±1.7a	16.7±2.1b	16.2±2.3b	17.1±2.4b	<0.05
Cv final (%)	31.5±2.8	30.0±2.3	30.3±2.4	30.2±2.2	>0.05
Tc (%)	21.8±1.7b	17.7±1.2a	17.3±1.3a	17.8±1.2a	<0.05
Tm (%)	15.0±1.7b	11.3±1.5a	10.0±1.4a	11.1±1.2a	<0.05
Ts (%)	64.0±3.4a	71.1±2.6b	74.0±2.8b	70.9±2.4b	<0.05
Cj (g/j)	0.12± 0.02a	0.17±0.02b	0.17±0.02b	0.18±0.02b	<0.05
Taca	3.77±0.18	3.61±0.14	3.59±0.16	3.57±0.17	>0.05

Expérience 2

Étude comparative de la survie et de la croissance entre les cages aménagées et les cages non aménagées pour différentes densités de mise en charge (100, 200, 500 et 1000 individus/m^3):

En fin d'élevage, les variations du poids moyen final (Pmf), du coefficient de variation du poids (Cv) et du taux de survie (Ts) en fonction des différentes densités de mise en charge entre les deux types de cages étudiées se font dans le même sens (Fig. 2), avec: (1) des Pmf élevés, (2) des Cv un peu plus faibles et (3) des Ts élevés dans les cages aménagées. Contrairement au coefficient de variation du poids (Ancova, $p>0.05$), le poids moyen final et le taux de survie sont plus hauts (Ancova, $p<0.05$) dans les cages aménagées (Tableau 4). Pour chacun des deux types de cages étudiées, aucune différence significative (*Post Hoc*: LSD, $p>0.05$) n'a été observée entre les Pmf obtenus aux densités de 100, 200, 500 et 1000 individus/m^3. Par contre, les valeurs de Cv et de Ts obtenues à ces densités sont différentes (*Post Hoc*: LSD, $p<0.05$) les unes des autres: les valeurs les plus intéressantes ont été enregistrées à la densité de 100 individus/m^3 et les moins intéressantes à celle de 1000 individus/m^3. Dans les cages non aménagées, le Pmf ne présente pas de liaison avec la densité de mise en charge. Par contre, le Cv et le Ts sont fortement liés à ce paramètre (Tableau 5). Les relations observées entre la densité de mise en charge et le Pmf, le Cv ou le Ts en cages non aménagées sont similaires à celles notées dans les cages aménagées.

TABLE 4

Résultats de la comparaison (analyse de covariance: Ancova) de la variation du poids moyen final (Pmf), du coefficient de variation du poids (Cv final) et du taux de survie (Ts) en fonction de la densité de mise en charge de *Heterobranchus longifilis* (poids initial: 0.9g) élevé pendant 90 jours en cage flottante sans système de refuge et en cage flottante avec système de refuge. Les valeurs de *p* marquées de (*) indiquent des différences significatives.

Paramètres	dl Effet	MC Effet	dl Erreur	MC Erreur	F	*p*
Pmf	1	72.4538	21	6.1038	11.8702	0.0024*
Cv final	1	13.5	21	104.8943	0.1287	0.7234
Ts	1	494.1338	21	66.8935	7.3869	0.0129*

TABLE 5

Résultats de la régression linéaire du poids moyen final (Pmf), du coefficient de variation du poids (Cv final) et du taux de survie (Ts) en fonction de la densité de mise en charge de *Heterobranchus longifilis* (poids moyen initial 0.9g) après 90 jours d'élevage en cage flottante sans système de refuge et en cage flottante munie de système de refuge (bandelette plastique blanche). Les valeurs de *p* marquées de (*) indiquent des liaisons significatives.

Traitements	Paramètres	n	R^2	F^1_{10}	*p*
Sans refuge	Pmf	12	0.0299	0.31	0.5907
	Cv final	12	0.7425	28.84	0.0003*
	Ts	12	0.8285	48.32	0.0001*
Avec refuge	Pmf	12	0.0001	0.01	0.996
	Cv final	12	0.7315	27.25	0.0001*
	Ts	12	0.8394	52.27	0.0001*

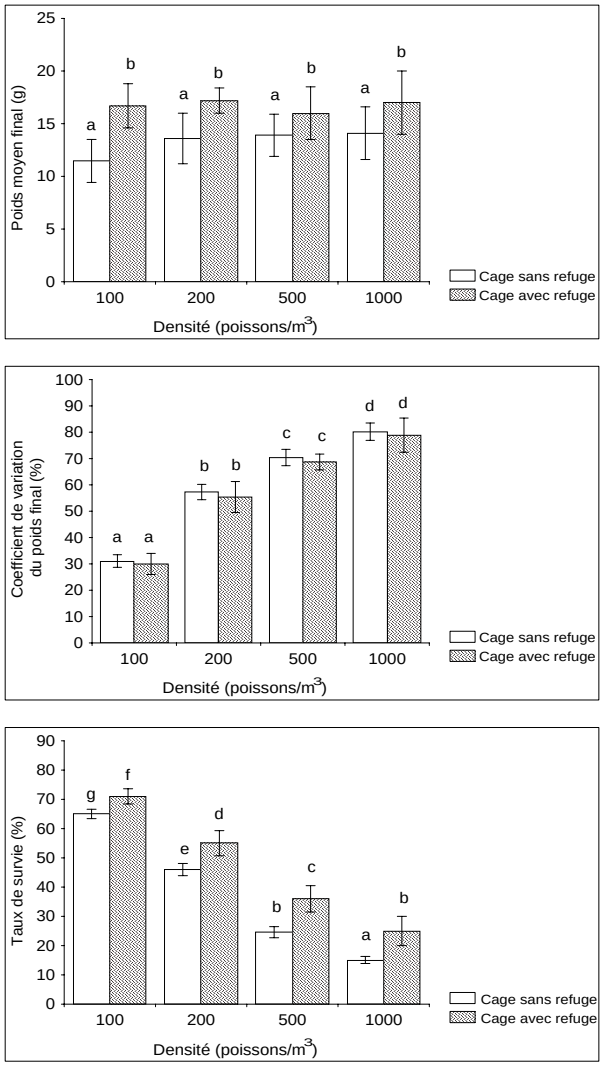

Fig. 2. – Influence de la densité de mise en charge (100, 200, 500 et 1000 individus/m^3) sur les paramètres de survie et de croissance des alevins de *Heterobranchus longifilis* (Poids moyen initial 0.9g) élevés pendant 90 jours en cages flottantes munies de système de refuge et en cages flottantes sans système de refuge. Pour chaque paramètre, des lettres différentes au dessus des histogrammes indiquent une différence significative $p<0.05$ (*Post Hoc*: LSD). Les barres verticales représentent les écarts entre réplicats n=3.

Expérience 3

Étude comparative de la survie et de la croissance entre les cages aménagées et les cages non aménagées pour des alevins de poids moyens initiaux différents (13.2±1.3g, 25.2±2.5g, 40.3±4.0g et 60.3±6.0g):

Après 60 jours d'élevage, pour les alevins de poids compris entre 13.2 et 29.2g, les valeurs les plus intéressantes de Pmf, de Ts et de Cv ont été enregistrées dans les cages aménagées (Tableau 6). Chez les poissons de Pmi égal à 13g, les paramètres (Pmf, Cv et Ts) enregistrés présentent des différences significatives (Test de Student, $p<0.05$). Pour les poissons de poids variant entre 25.2 (Pmi) et 46.0g (Pmf), seul le Ts en cages aménagées (plus intéressante valeur: 94.0±1.0%) est statistiquement différent (Test de Student, $p<0.05$) de celui enregistré en cages non aménagées. Chez les individus de 40.3 (Pmi) à 65.0g (Pmf), la comparaison des paramètres (Pmf, Cv et Ts) enregistrés dans les cages aménagées à ceux obtenus dans

TABLE 6

Paramètres de survie et de croissance en fonction du poids moyen initial (Pmi) de mise en charge de *Heterobranchus longifilis* élevé pendant 60 jours en cage flottante munie de système de refuge (bandelette plastique blanche) dans le lac de barrage d'Ayamé (Pmf=poids moyen final, Cv=coefficient de variation et Ts=taux de survie). Pour chaque Pmi, dans une même colonne, les valeurs portant des lettres différentes sont significativement différentes (Test de Student, $p<0.05$); les écarts-types ont été calculés entre triplicats.

Pmi (g)	Cage flottante	Pmf (g)	Cv final (%)	Ts (%)
13.2±1.3	Sans refuge	23.1±2.4a	66.0±3.0b	80.0±3.6a
	Avec refuge	29.2±2.6b	59.0±2.6a	89.0±4.0b
25.2±2.5	Sans refuge	45.0±2.4a	56.0±3.0a	88.0±3.0a
	Avec refuge	46.0±2.6a	54.0±2.6a	94.0±1.0b
40.3±4.0	Sans refuge	65.0±2.1a	47.0±2.5a	92.0±3.4a
	Avec refuge	64.0±2.6a	49.0±2.3a	93.0±3.0a
60.3±6.0	Sans refuge	92.0±1.1a	43.0±2.5a	95.0±2.6a
	Avec refuge	85.0±2.6b	44.0±2.0a	89.0±2.0b

les cages non aménagées n'a révélé aucune différence significative (Test de Student, $p>0.05$). A l'exception du Cv (Test de Student, $p>0.05$), le suivi du Pmf et du Ts pour des poissons de poids compris entre 60.3 et 92.0g, indique une différence significative (Test de Student, $p<0.05$) entre les lots de poissons élevés en cages aménagées et ceux élevés en cages non aménagées: les valeurs les moins intéressantes (Pmf: 60.3±6.0g; Ts: 89.0±2.0%) ont été notées dans les cages aménagées.

DISCUSSION

D'une façon générale, le système de refuge proposé dans cette étude s'est révélé efficace pour l'amélioration du taux de survie des alevins de *H. longifilis* de poids moyen compris entre 0.9 et 17.1g quelle que soit la densité de mise en charge (100, 200, 500 et 1000 individus/m^3). Une telle amélioration de la survie a déjà été notée suite à des aménagements intervenus dans le milieu d'élevage. En effet, en introduisant un système de refuge constitué de touffes de nylon dans des ''cages enclos'' implantées en milieu lagunaire, LEGENDRE et al. (1991) sont parvenus à faire passer la survie de *H. longifilis* (Pmi: 1.8mg; Pmf: 369mg; durée d'élevage: 14 jours) de 7 à 15% en moyenne avec des valeurs pouvant atteindre 50%.

Les mortalités obtenues lors de la présente étude sont surtout (expérience 1) le fait du comportement cannibale des alevins de *H. longifilis*. Il est bien connu que chez certains poissons-chats, la mortalité due à ce comportement est liée à la différence de tailles au sein de la population (HECHT & APPELBAUM, 1988; APPELBAUM & KAMLER, 2000). Par conséquent, l'amélioration de la survie suite à l'introduction d'un système de refuge dans le milieu d'élevage peut être liée à la capacité de certains individus (éventuellement les plus vulnérables) à se réfugier à l'intérieur du nuage constitué par les bandelettes plastiques.

L'estimation du risque de cannibalisme au sein d'une population a été abordée dans plusieurs travaux (APPELBAUM & KAMLER, 2000; BARAS & D'ALMEIDA, 2001; BROWN & BRAITHWAITE, 2004). Ce risque est d'autant plus grand que la variabilité de taille est élevée (APPELBAUM & KAMLER, 2000). Dans la présente étude, cette variabilité a été estimée au moyen du coefficient de variation du poids. Dans les expériences 1, 2 et 3 [excepté le lot de poissons de poids variant entre 13.2 (Pmi) et 29.2g (Pmf)], aucune différence ($p>0.05$) n'a été enregistrée entre les coefficients de variation du poids des deux types de cages testées. Malgré cette variabilité de poids comparable, la survie a été meilleure chez les individus élevés en cages aménagées. Dans ces dernières, les individus les plus vulnérables et/ou les plus petits ont probablement utilisé le nuage de bandelettes comme refuge pour échapper à la prédation. Ces observations pourraient par conséquent expliquer les valeurs élevées de survie enregistrées en présence du système de refuge.

Lors du suivi des paramètres de survie en fonction du poids moyen initial des poissons, les résultats ont montré que l'utilisation du système de refuge testé a influencé significativement et positivement le taux de survie des *H. longifilis* de poids moyen compris entre 0.9 (Pmi) et 29.2g (Pmf). Cette influence du système de refuge est négative pour les poissons de poids moyens compris entre 60.3 (Pmi) et 92g (Pmf). Ces résultats tendent à confirmer que l'efficacité du système de refuge testé dans la présente étude dépend de la taille des poissons.

Le système de refuge testé a également influencé le poids moyen final des poissons. En effet, comparativement aux cages témoins, l'utilisation du système de refuge (cages aménagées) au cours de l'élevage des poissons de poids moyen inférieur ou égal à 29.2g a produit un effet bénéfique. Lors de l'utilisation d'un système de refuge constitué de touffes de nylon, LEGENDRE et al. (1991) ont également noté une amélioration du poids moyen final (de 1.8 à 369mg) chez *H. longifilis* élevé pendant 14 jours à une densité de mise en charge de 16 alevins/litre. Chez un autre poisson-chat (*Clarias gariepinus*) de poids moyen initial égal à 0.8g, HOSSAIN et al. (1998) ont aussi noté une amélioration du poids moyen final avec l'utilisation d'un système de refuge fait de matière plastique. Cette amélioration de la croissance avec l'introduction de système de refuge dans le milieu d'élevage pourrait s'expliquer par plusieurs facteurs: la réduction du stress avec l'évitement des prédateurs (WOODLEY & PETERSON, 2003) et l'augmentation du temps des périodes d'inactivités (HECHT & APPELBAUM, 1988). Chez les poissons, il existe une relation inverse entre l'énergie utilisée pour couvrir les activités (métabolisme de base, locomotion, quête de nourriture, actions d'évitement des prédateurs et coût d'entretien) et celle investie pour la croissance (CALOW, 1985; APPELBAUM & KAMLER, 2000). Ainsi, lorsque le temps de repos est prolongé, une partie de l'énergie destinée à la locomotion, à la quête de nourriture et à l'évitement des prédateurs est utilisée pour la croissance. Les taux de croissance intéressants enregistrés lors de l'élevage des larves de *C. gariepinus* en condition continue d'obscurité ont été justifiés par un faible taux d'énergie utilisée pour la locomotion, ces larves étant moins actives dans ces conditions (APPELBAUM & KAMLER, 2000). Une telle influence de l'augmentation des périodes de faibles activités sur la croissance a également été observée chez des larves de cette espèce en présence de système de refuge (HECHT & APPELBAUM, 1988). Par conséquent, il est donc possible que l'amélioration de la croissance enregistrée au cours du présent travail (pour des poissons de poids moyen inférieur ou égal à 29.2g) soit le résultat d'une relative longue période de faible activité passée à l'intérieur du nuage constitué par les bandelettes du système de refuge. En plus du rôle de refuge des bandelettes, leur présence pourrait aussi avoir amélioré la croissance des poissons en fournissant une source non négligeable d'organismes se développant sur les bandelettes servant de substrat à un fouling. Chez les individus de plus grande taille (expérience 3: poids moyen initial de 25, 40 et 60g) le système de refuge testés n'a pas d'effet améliorateur sur la croissance. Il est donc possible que les bandelettes du système de refuge telle que présentées constituent un obstacle pour ces individus du fait de leur taille. Dans ces conditions, en plus de ce rôle d'obstacle, le système de refuge réduit de 25% (volume occupé par le nuage de bandelettes) l'espace vital de ces poissons par rapport aux individus maintenus en cages sans système de refuge. Une telle réduction de l'espace vital pourrait conduire à une situa-

tion de confinement dont l'une des conséquences est le développement d'un état de stress. Ce qui pourrait, d'après PICKERING (1993), provoquer une diminution de la prise de nourriture. Cette chute de la consommation d'aliment a pour conséquence la réduction de la croissance (MÉLARD, 1986).

Les travaux de LEGENDRE (1991) ont montré que de meilleurs taux de croissance de *H. longifilis* pouvaient être obtenus dans des structures sans système de refuge (étang, enclos, cage-enclos): chez des juvéniles de poids moyen 0.77g élevés en étang pendant 70 jours à la densité de 10 individus/m^2, une croissance journalière de 0.30g/j a été observée; chez des poissons de poids moyen supérieur à 10g, cet auteur a enregistré des gains de poids journaliers variant entre 2.74 et 3.90g/j en cage-enclos (3 à 8.5 individus/m^2), 3.52g/j en étang (0.7 individus/m^2), 4.27g/j en enclos (2 individus/m^2) et 3.24g/j en bassin en béton (7.5 individus/m^2). Les valeurs obtenues dans ces structures seraient surtout dues au fait qu'elles ont en commun une productivité naturelle importante composée de détritus organiques, débris végétaux et animaux, graines, fruits, coquilles de gastéropodes, larves de batraciens, insectes, vertébrés aquatiques et proies planctoniques (GILLES et al., 2001). Selon MICHA (1973) et OTÉMÉ et al. (1996), *H. longifilis* est un poisson omnivore à tendance carnassière. Par conséquent, dans ces structures d'élevage, en plus de l'aliment artificiel, ce poisson bénéficie des ressources naturelles. Les espèces élevées en cages flottantes par contre ne dépendent que de l'apport d'aliment exogène (COCHE, 1978).

En définitive, le système de refuge proposé dans la présente étude pour l'amélioration de la survie et de la croissance des alevins de *H. longifilis* élevés en cage flottante s'est révélé efficace. L'efficacité de cette structure reste toutefois limitée aux individus de taille inférieure ou égale à 29.2g.

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Is duration of organic management reflected on nematode communities of cultivated soils?

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ABSTRACT. The aim of this study was (a) to explore long-term responses of soil nematodes to the conversion from conventional to organic cultivation and (b) to differentiate them from the short-term responses to seasonal agricultural practices. Nematode communities were studied in terms of trophic and generic structure, life strategy and diversity, in asparagus cultivations along a gradient from conventional to organic under conversion (1 and 2 years), and certified organic (4 and 5 years). Samplings throughout the year were conducted with respect to seasonal agricultural practices.

Changing management regime from conventional to organic cultivation seemed to initiate successional long-term changes in nematode communities, such as the gradual decline of phytoparasites in favour of bacterivores and fungivores, the gradual decrease of PPI, the increase of diversity. Alterations in the generic structure of the community were also revealed, driven mostly by the opposite trends of changes in abundance of *Helicotylenchus* vs. *Heterocephalobus*. Seasonal agricultural practices appeared to induce short-term responses of functional guilds of low colonizer-persister values (c-p 1 and 2) mostly, and were reflected in nematode indices, such as Maturity Index, Plant Parasitic Index, Enrichment Index, Channel Index but not Structure Index. Nematode responses at the generic level to seasonal agricultural practices seemed less intense than the ones imposed by changing management regime, and in the case of conventional cultivation they were almost entirely masked.

KEY WORDS : asparagus, conventional agriculture, conversion to organic, diversity, *Helicotylenchus*, *Heterocephalobus,* nematode indices

INTRODUCTION

In the past 20 years, considerable attention has been paid to nematodes, demonstrating that these ubiquitous members of the soil community reflect change in ecological structure and function of soils in ways more predictable and efficient than for other soil flora or fauna (FISCUS & NEHER, 2002). The ecological significance of nematodes lies in their high abundance and diversity, variety of trophic types and reproductive strategies (YEATES, 2003). They have been used to evaluate soil conditions under different crops (WIDMER et al., 2002), under various agricultural practices, such as tillage (FU et al., 2000), fallow farming (VILLENAVE et al., 2001), use of pesticides (LIANG et al., 2001), fungicides (VILLENAVE et al., 2004) or herbicides (YEATES et al., 1999), and to distinguish the effects of physical and chemical disturbance of cultivated soils (FISCUS & NEHER, 2002).

Furthermore, nematodes have been used for evaluating soil quality under organic and conventional agriculture (FRECKMAN & ETTEMA, 1993; FERRIS et al., 1996; NEHER, 1999; NEHER & OLSON, 1999; MULDER et al., 2003; GARCÍA-ÁLVAREZ et al., 2004 among others). However, only a few studies compare agroecosystems managed organically for different time periods, and are mostly confined to comparisons of crop yields or to soil parameters other than nematodes (FRIEDEL & GABEL, 2001; MARTINI et al., 2004; MONOKROUSOS et al., 2006). To our knowledge, the only studies relating nematode communities to duration of organic management are those of BERKELMANS et al. (2003) who monitor changes of nematode communities in sites managed organically from 4 to 12 years, and of VAN DIEPENINGEN et al. (2006) who compare conventional to certified organic systems of less and more than five years of organic management. Thus, there is a lack of data regarding possible trends of succession within communities initiated after changing management regime from conventional to organic.

The main goal of this study was, therefore, to explore responses of soil nematodes along a 5 year gradient from conventional to organic cultivation. According to the Regulation 2092/91/EEC, the period of conversion from a conventional to an organic production system is at least two years for annual crops and three for perennial ones. The agricultural systems included in our study were conventional, organic under conversion (1 and 2 years) and certified organic (4 and 5 years) cultivations of *Asparagus officinalis*. We selected them in order to minimize the effect of other factors that are known to affect nematode populations even more than agricultural practice *per se*, such as the cultivated species and duration of cultivation (YEATES et al., 1999) and the soil type (VAN DIEPENINGEN et al., 2006). Moreover, asparagus cultivations are perennial and not additionally stressed by annual plantings, and thus the history of agricultural practices is better reflected on soil conditions. Nematode communities were studied in terms of trophic and generic structure, life strategy and diversity.

Apart from changing management regime from conventional to organic, seasonal agricultural practices, input of fertilizers (conventional or organic), mechanical disturbance of soil, crop harvesting etc, are shared between management types, constituting periodic disturbances to the soil ecosystem. These disturbances are quite impor-

tant for shaping nematode communities. The effect of the one may mask the effect of the other (FISCUS & NEHER, 2002), but most importantly they may mask the progressive effect of the transition from conventional to organic management. Thus, a second goal of our study was to explore short-term nematode responses to seasonal agricultural practices and compare them to the long-term responses to changing management regime. This was achieved by conducting samplings in periods following all steps within the annual crop growing cycle of asparagus.

MATERIALS AND METHODS

Sites and sampling

The study area is a cultivated plain of Northern Greece, at about 60km north west of Thessaloniki (7.5m *a.s.l.*), where asparagus is traditionally one of the main products. Due to higher price of organic agricultural products and financial support by the state, during the last decade a lot of asparagus cultivations were converted to organic. Soils are classified as luvisol (FAO) with a clay-loam texture (37% sand, 33% silt, 30% clay). The climate is transient between mediterranean and continental. Mean annual precipitation for a 10-year period was 485mm and mean annual temperature 14°C. January was the coldest month (4°C) with the highest precipitation (58mm), while the warmest (24°C) and driest (16mm) month was July.

The experimental plots of our study belonged to agroecosystems with different management history, i.e. conventional, organic under conversion and certified organic cultivations of the perennial *Asparagus officinalis*. Conventional sites covered about 9,000m^2 and were cultivated with asparagus for more than 6 years. Organic ones were conventionally cultivated for several years before conversion to organic farming, and covered in total about 30,000m^2 of the wider study area. At the start of the study the certified organic sites were managed organically from 4 to 5 years [O4, O5], while the transitional organic sites were managed organically from 1 to 2 years [O1, O2]. Asparagus cultivations, either organic or conventional, are not tilled, but during February, i.e. one month before the beginning of the harvest season, the soil between field rows is mounded up over the asparagus plants, for producing white spears. The soil mounds are smoothed away during June, i.e. after the end of harvest. Thus, all plots are subject to the same physical disturbance and differ only regarding the type of fertilizers and weed control. Fertilization takes place in summer, with manure and licensed organic fertilizers in one case and synthetic fertilizers in the other (Table 1). Regarding weed control, which also takes place in summer, it is done with handhoeing in case of organic cultivation, while in conventional cultivation it is done once every summer by means of Linuron for broadleaved grasses (0.2 l /1,000m^2) and Fluazifop-p-butyl for grass weeds (0.2 l /1,000m^2).

Our sampling scheme represented a full factorial design of 5 agricultural systems x 4 dates x 4 replicate plots. The latter were randomly dispersed covering 250m^2 each. At each plot we took a composite sample of 10 soil cores 2cm in diameter and 20cm in depth. Samples were taken in the rows of the asparagus plantations, and more specifically close to the edge of them, so that regardless the seasonal soil mounding, all year samples corresponded to the same soil depth relative to the cultivated plants. Soil sampling was conducted four times throughout a year; in March (asparagus spears start growing and the harvest begins), in May (end of harvest), in October (the fields are left with no agricultural activities for 2 – 3 months) and in December (two weeks after cutting of the aboveground parts of asparagus plants which are then left in the field to decompose). The time span between samplings was long in relation to nematode life span, so that nematode population dynamics decouple nematode counts across time, creating sufficiently independent data.

TABLE 1

Fertilizers in organic and conventional cultivation. Total amounts [in brackets] and time of application are indicated. All fertilizers used in organic cultivation are permitted for organic agriculture.

Organic	
Manure [1T/1,000m^2]	August (once every two years)
Organic fertilizer (Bioazoto, 12% N 14% C) [200kg/1,000m^2]	May (once every year)
Mineral Potassium Sulphate [550Kg/1,000m^2]	
Organic fertilizer (Dermafert, N P K 8-7-7 + 2MgO + 8SO_3+17 C) [1,000kg/1,000m^2]	July (once every year)
Conventional	
Synthetic fertilizer (Hydrocomplex supra N P K 6-15-25 + 3MgO + 30SO_3) [100Kg/1,000m^2]	July (once every year)

Nematodes were extracted from 150mL of each composite soil sample. Before taking this subsample, the soil was gently mixed by hand and soil aggregates were broken up. For extraction, we used the modified Cobb's sieving and decanting method proposed by S'JACOB & VAN BEZOOIJEN (1984), according to which a cotton-wool filter is used in the last step. After counting total abundance of nematodes, we fixed them with formaldehyde 4%. Later on from each sample we selected randomly at least 150 nematodes and identified them to the genus level in most cases, using the identification key of BONGERS (1994).

Soil bulk density and pH was measured once, at the beginning of samplings. The former was not found to differ among study sites, ranging from 1.06 to 1.24g/cm^3. Values of pH decreased gradually and significantly from 7.99 in [C] to 7.94, 7.90, 7.83 and 7.51 in [O1], [O2], [O4] and [O5] respectively. On each sampling occasion, soil water content (% dry weight) was also estimated from each soil sample taken. It was found to differ seasonally, being higher in December (24.22%) and lower in May (20.36%).

Nematode indices

Nematode taxa were assigned to trophic groups according to YEATES et al. (1993), classified along the colonisation-persistence gradient (c-p values) following BONGERS

(1990) and BONGERS & BONGERS (1998), and arranged to functional guilds (portions of particular trophic groups exhibiting the same c-p value) according to BONGERS & BONGERS (1998) and FERRIS et al. (2001).

The maturity index (MI) for free living nematodes (c-p from 1 to 5) and the plant parasitic index (PPI) for plant feeding nematodes, both indicating the successional stage of communities, were calculated according to BONGERS (1990) as $\Sigma v_i p_i$, where v_i is the c-p value of taxon i and p_i the proportion of the taxon in the nematode community. The Enrichment index (EI), the Structure index (SI) and the Channel Index (CI), were calculated according to FERRIS et al., (2001). EI and SI, which provide location of the food web along the enrichment and the structure trajectory, were calculated according to the formulas $EI = 100 \times (e/(e+b))$ and $SI = 100 \times (s/(s+b))$. The b component refers to the nematode functional guilds that indicate basal characteristics of the food web, namely the bacterivores with c-p 2 value (Ba2) and the fungivores with c-p 2 value (Fu2), and was calculated as $\Sigma k_b n_b$, where k_b are the weightings assigned to the Ba2 and Fu2 functional guilds and n_b are the abundances of nematodes in those guilds. The e and s components were calculated similarly, using those guilds indicating enrichment (Ba1, Fu2) and structure (Ba3–Ba5, Fu3–Fu5, Om3–Om5, Pr2–Pr5), respectively. Finally CI which indicates the predominant decomposition pathway, was calculated as $CI = 100 \times (0.8Fu2/(3.2Ba1+0.8Fu2))$.

Data analysis

All analyses aim to estimate and compare the nematode responses to changing management regime and to seasonal agricultural practices, in terms of trophic structure, nematode indices, generic structure and composition and diversity.

For analyzing frequencies of the different nematode trophic groups at the different agricultural systems, we used the log-linear analysis of count data of categorical variables. This method fits log-linear models to the combinations of categorical variables, herein trophic group and site, to find out possible associations between them. More specifically, it estimates the randomly expected frequency of every combination of trophic group and site and compares it to the observed frequency.

For testing differences of nematode indices due to the long-term effect of changing management regime as well as the short-term effect of seasonal agricultural practices, we used two-way ANOVA (site x date). LSD post hoc comparisons where performed, when significant differences were revealed. Prior to analyses, data were examined for normality, homogeneity of variance (Levene's test) and independence between variance and mean. Since values of nematode indices are bounded, an arc-sinus transformation was used. Kruskal-Wallis test was used in the case where the prerequisites of normality were not met even after transformation.

In order to compare the progressive effects of changing management regime to those of seasonal agricultural practices on the generic structure of nematode communities, all samples and nematode genera abundances were ordinated by means of correspondence analysis (CA), while sample ordination scores for Axis 1 and Axis 2 were analyzed by MANOVA (site x date). When significant differences were revealed, post hoc comparisons were performed by means of LSD-test. Abundances of nematode genera were log+1 transformed prior to CA, while prior to MANOVA data were examined for normality, homogeneity of variance (Levene's test) and independence between variance and mean.

For assessing the diversity of nematode communities, we used the method of diversity ordering proposed by RENYI (1961). Renyi's parametric index of order a shows varying sensitivity to the rare and abundant species of a community, as the scale parameter a changes (RICOTTA, 2000). For each community it provides a profile of the most widely used diversity indices. For a=0, the index equals log species number, for a=1, it equals Shannon's index, for a=2, it equals Simpson's index. For a tending to infinite, the index is most sensitive to the abundant species of a community. Thus, when diversity profiles differ in the range of low a values, this is due to the number of species. In the range of high a values, differences between communities are due to presence of abundant species. When diversity profiles intersect, the communities may be ordered differently by different diversity indices.

In order to study changes of generic composition, we used the IndVal (Indicator Value) method of DUFRENE & LEGENDRE (1997). This method assigns indicator (characteristic) species to a site or a group of sites, on the basis of species relative abundance and relative frequency of occurrence in the samples. For the classification of sample units required by IndVal, we used a complete linkage hierarchical classification tree based on 1-Pearson r distance measure. The statistical significance of the species indicator values was also evaluated by the IndVal program by means of a randomization procedure.

For log-linear analysis, ANOVA, MANOVA, Kruskal-Wallis and tree clustering we used the SPSS software package (version 11). For Correspondence analysis we used CANOCO (version 3.10) software package (TER BRAAK, 1988), while diversity ordering was performed by means of DivOrd (TOTHMERESZ, 1995).

RESULTS

Trophic structure

Bacterivores were the dominant trophic group in all organic plots comprising over 48% of the nematode communities (Table 2). In the case of conventional plots the dominant trophic group was that of phytoparasitic nematodes (50%), followed by bacterial feeders (30%). Predators and omnivores accounted for less than 2% in all cases. Total abundance ranged from 425 to 1345 ind./100ml soil. Abundance changes did not exhibit a specific trend, masking possible interactions between trophic groups and sites, which are apparent from the% contribution of each group. Therefore, in order to test statistically the frequency of appearance of each group at each site we proceeded to log-linear analysis.

Associations between a trophic group and a site, explored by log-linear analysis, are presented in Fig. 1. Bacterivores and non parasitic plant feeders seemed to appear less frequently at [C] and [O1], being more frequent at older organic cultivations [O4, O5]. An exactly opposite trend

was exhibited by phytoparasites. Fungivores appeared more frequently at organic plots instead of [C], not exhibiting though gradual changes from recent to older organic cultivations. Predators were more frequent at [O4] and [O5], while omnivores at [C] and [O1]. However, contribution of these trophic groups was very low and therefore sampling errors as indicated by confidence intervals were very high to arrive at a secure conclusion about their response.

TABLE 2

The contribution of individual trophic groups (%) and the total nematode abundance (±SE) in the studied agricultural systems. **[C]:** conventional, **[O1], [O2], [O4], [O5]:** managed organically for 1, 2, 4 and 5 years respectively.

	[C]	[O1]	[O2]	[O4]	[O5]
Bacterivores (%)	29.78 (5.31)	48.23 (5.91)	50.61 (4.25)	51.66 (3.57)	61.01 (2.86)
Fungivores (%)	10.48 (1.59)	23.69 (4.66)	19.41 (2.55)	25.59 (2.16)	17.40 (2.09)
Phytoparasites (%)	49.98 (5.30)	19.06 (3.78)	16.29 (4.46)	7.63 (2.00)	9.85 (2.46)
Non parasitic plant feeders (root hair & algal feeders) (%)	7.17 (1.91)	7.52 (0.87)	12.11 (1.98)	13.41 (1.97)	10.33 (1.69)
Predators (%)	1.13 (0.38)	1.19 (0.35)	0.72 (0.29)	1.63 (0.45)	1.34 (0.28)
Omnivores (%)	1.46 (0.55)	0.30 (0.17)	0.86 (0.3)	0.08 (0.08)	0.07 (0.07)
Total abundance (ind./100ml soil)	1068 (180)	1345 (244)	1018 (193)	425 (90)	778 (128)

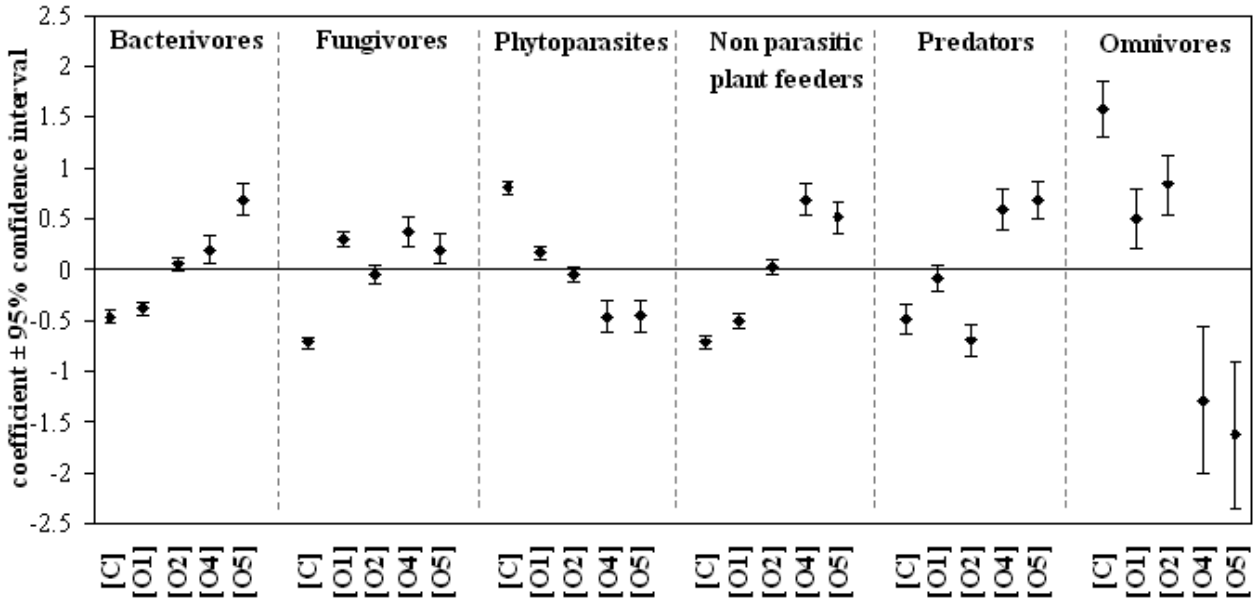


Fig. 1. – Coefficients of the Log-Linear model for each combination of trophic group and site. When the confidence intervals of the coefficient include 0 the nematode population of the specific trophic group at the specific site does not differ from the overall average. Positive values of the coefficient indicate that the specific trophic group appears at the specific site more frequently than randomly expected, while the inverse happens for negative values. Codes as in Table 2.

Nematode indices

Mean values of MI, PPI, EI, SI and CI at the different sites and seasonal samplings are presented in Table 3, together with the results of two-way ANOVA (site x date). Significant changes regarding sampling site were observed only in the case of PPI, which increased gradually from the older to the recent organic and further to conventional cultivation. Regarding sampling date, all indices except SI displayed significant differences. MI was higher in October, i.e. in a period long after any agricultural practice. PPI was higher in December, i.e. when the above ground parts of asparagus plants were cut and left in the field to decompose. EI was higher in March, i.e.

in the beginning of the growing season, shortly after the soil was mounded up. CI was lower in March and May, i.e. during the whole harvest season.

Generic structure

The ordination of all samples along the two first axes of Correspondence Analysis is depicted in Fig. 2, while the results of MANOVA (site x date) on Axis 1 and Axis 2 ordination scores are given in Table 4. The effects of changing management regime on generic structure of the nematode community were significant at both axes, while those of seasonal agricultural practices were significant only along the second axis. Samples from [C] were clearly and significantly distinct from [O4] and [O5] samples, forming a gradient from conventional to recent and further to older organic cultivations along the first axis. Samples taken during the harvest season when the soil was mounded, i.e. March and May, were ordinated on the lower half of the plot, differing significantly from October and December samples, which were ordinated mostly on the upper half corresponding to samplings carried out during the post-harvest season when soil was flat. Because of these seasonal differences in generic structure, we provide separate results for the harvest and the post-harvest season in all subsequent analyses of the data.

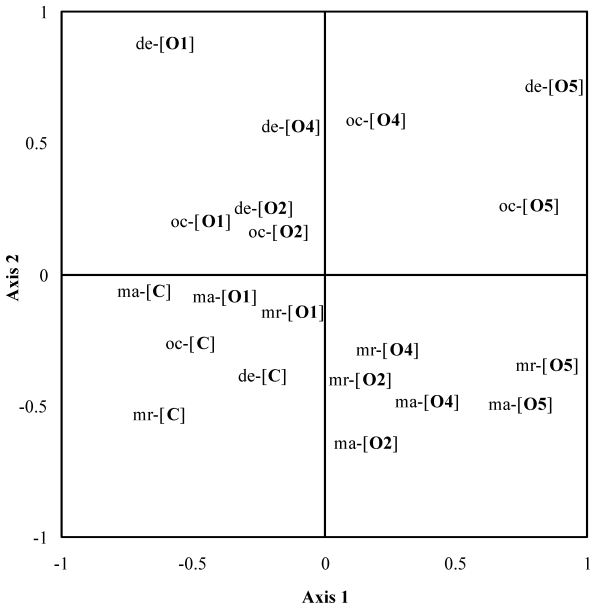


Fig. 2. – Correspondence Analysis two axes plane for samples from different sites and sampling dates. Each point represents mean values of sample scores for each site at each sampling date. Site codes as in Table 2. (mr: March, ma: May, de: December, oc: October).

TABLE 3

Mean values (±SE) of Maturity Index, Plant Parasitic Index, Channel Index, Enrichment Index, Structure Index and results of two-way ANOVA (site x date) or Kruskal-Wallis test (for SI). Superscripts a, b, c, d indicate differences revealed by LSD-test, (*: P<0.05, **: P<0.001, MS: Mean square). Codes as in Table 2.

	MI	PPI	CI	EI	SI
SITE (4 d.f)					
[C]	1.92 (0.07)	2.85 (0.04)^{d}	31.77 (6.17)	60.77 (4.38)	27.31 (6.25)
[O1]	1.85 (0.04)	2.64 (0.05)^{c}	35.51 (7.49)	58.83 (2.87)	11.63 (2.69)
[O2]	1.83 (0.05)	2.48 (0.08)^{c}	34.11 (6.50)	58.11 (4.44)	13.68 (3.30)
[O4]	1.86 (0.04)	2.29 (0.06)^{b}	35.82 (5.58)	56.24 (3.49)	11.88 (2.51)
[O5]	1.77 (0.04)	2.06 (0.03)^{a}	20.69 (3.48)	61.79 (3.95)	11.66 (2.67)
MS	0.07	2.59	0.10	0.02	
F	2.07	35.40	1.31	0.53	*H*=4.17
P	NS	**	NS	NS	NS
DATE (3 d.f)					
March	1.75 (0.05)^{a}	2.42 (0.06)^{a}	17.59 (3.08)^{a}	70.26 (2.77)^{c}	19.93 (4.18)
May	1.84 (0.04)^{a}	2.45 (0.08)^{a}	22.67 (3.80)^{a}	58.23 (3.23)^{b}	13.78 (3.48)
October	1.95 (0.02)^{b}	2.38 (0.09)^{a}	46.75 (5.92)^{b}	48.13 (2.87)^{a}	14.29 (3.68)
December	1.85 (0.04)^{a}	2.60 (0.08)^{b}	39.30 (5.60)^{b}	59.96 (2.97)^{b}	12.93 (2.76)
MS	0.15	0.39	0.60	0.27	
F	4.72	5.34	7.58	8.89	*H*=1.47
P	*	*	**	**	NS
SITE x DATE (12 d.f.)					
MS	0.08	0.01	0.12	0.03	
F	2.65	1.33	1.56	1.05	
P	*	NS	NS	NS	

TABLE 4

Results of MANOVA (site x date) for Axis 1 and Axis 2 sample ordination scores. Superscripts a, b, c, d indicate differences revealed by LSD-test, (*: P<0.05, **: P<0.001, ***: P<0.0001, MS: Mean square). Codes as in Table 2.

overall effect	**Value**	**Error d.f.**	**Hypothesis d.f.**	**F**	**P**
Intercept	0.99	59	2	0.19	NS
SITE	0.21	118	8	17.47	***
DATE	0.51	118	6	7.85	***
SITE x DATE	0.52	118	24	1.93	*

univariate results	**SITE (4 d.f)**				**DATE (3 d.f)**			
	LSD	*MS*	*F*	*P*	*LSD*	*MS*	*F*	*P*
Axis 1	[C]^{a} [O1]^{a} [O2]^{b} [O4]^{c} [O5]^{d}	4.51	39.31	***		0.06	0.56	NS
Axis 2	[C]^{a} [O2]^{ab} [O5]^{bc} [O4]^{bc} [O1]^{c}	0.72	4.72	*	march^{a} may^{a} octob^{b} decemb^{b}	2.90	18.85	***

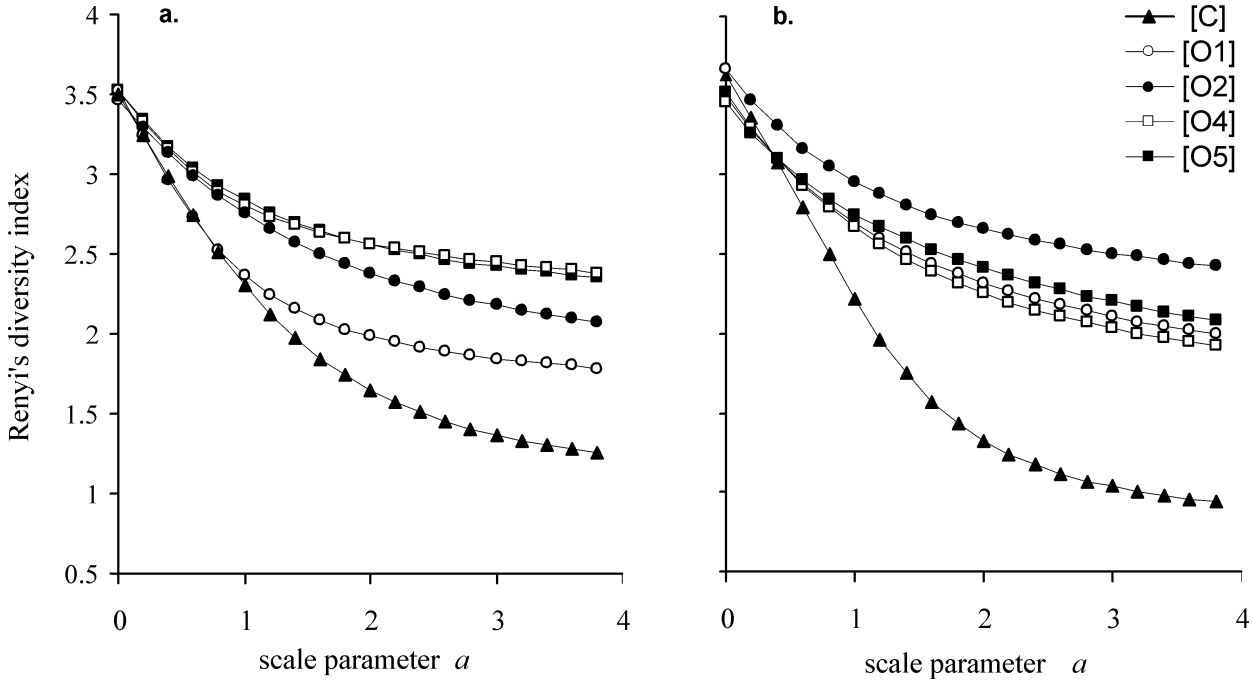
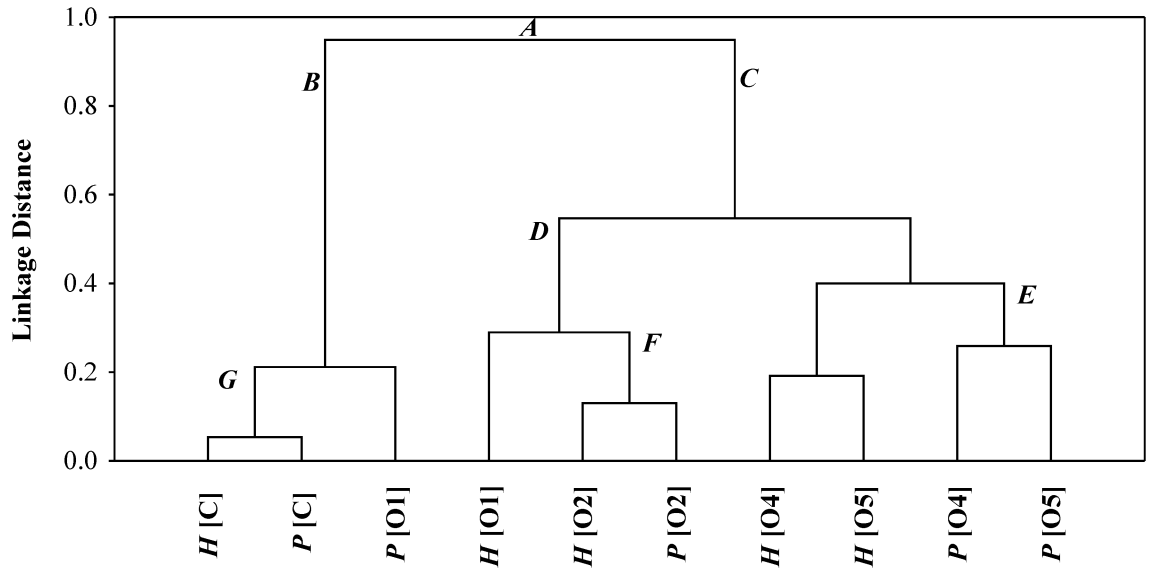


Fig. 3. – Diversity profiles of nematode communities in the studied agricultural systems during (a) the post-harvest season and (b) the harvest season. Codes as in Table 2.

The diversity profiles of the nematode communities for all studied systems are shown in Fig. 3. In the post-harvest season (Fig. 3a), diversity was higher in [O5], [O4] and gradually decreased to [O2], [O1] and further to [C], reflecting a gradient of a long-term management regime. In the harvest season (Fig. 3b), the diversity of recent organic plots increased, while [C] was even less diverse than in the previous season. In both seasons, differences of diversity were not due to the number of genera, since the profiles differed mainly in the range of high values of the scale parameter *a*. This means that differences of diversity were mainly due to the distribution of nematode numbers among genera. Indeed, the contribution of the most abundant genera of the organic sites did not exceed 22%. In [C] the community displayed a quite different structure due to the strong dominance of a single genus, namely *Helicotylenchus,* which accounted for 40% of the community in the post-harvest season. The strong dominance of this semiendoparasite in [C] was even higher (50%) in the harvest season, causing the observed decrease of diversity in this case.

A tree clustering of sites is presented in Fig. 4. Each site is represented twice; once for the post-harvest and once for the harvest season, coded as *P* and *H* respectively. Moreover, we provide the maximum indicator value of each genus at the site or cluster of sites to which it is assigned. Changing management regime from conventional to organic cultivation caused profound modifications in generic structure and composition, since organic plots were separated from conventional, regardless of season. The most recent organic plots constituted an exception since they were clustered either with the



A	IndVal
Acrobeloides (BA-2)	100
Aphelenchus (FU-2)	98.75
Chiloplacus (BA-2)	98.75
Filenchus (NP-2)	90
Eucephalobus (BA-2)	85
Aphelenchoides (FU-2)	75
Panagrolaimus (BA-1)	72.50
Malenchus (NP-2)	71.25
Mesorhabditis (BA-1)	71.25
Eumonhystera (BA-2)	67.50
Protorhabtitis (BA-1)	61.25
Rhabditidae 1 (BA-1)	52.50
Boleodorus (NP-2)	42.50
Paraphelenchus (FU-2)	42.50
Bursilla (BA-1)	41.25
Tylenchus (NP-2)	40
Diploscapter (BA-1)	31.25
Coarctadera (BA-1)	30
Cervidellus (BA-2)	28.75
B	
Helicotylenchus (PA-3)	76.31**
Tylenchorhynchus (PA-3)	50.80**
Eudorylaimus (PR-4)	32.28**
Cuticularia (BA-1)	14.13**
C	
Heterocephalobus (BA-2)	67.30**

D	IndVal
Drilocephalobus (BA-2)	30.87**
Ditylenchus (FU-2)	26.12**
E	
Thonus (PR-4)	22.66**
Diplogasteridae (BA-1)	8.11
F	
Pristionchus (BA-1)	26.53
G	
Zygotylenchus (PA-3)	12.50
***H* [C]**	
Laimydorus (OM-5)	36.98**
Plectus (BA-2)	12.50
Epidorylaimus (OM-4)	12.50
Discolaimidae (PR-5)	12.50
Basiria (NP-2)	10.53
Cephalobus (BA-2)	10.51
Acrobelophis (BA-2)	7.66
***P* [C]**	
Criconematidae (PA-3)	25
Teratocephalus (BA-3)	25
Rhabditidae 2 (BA-1)	21.08
***P* [O1]**	
Pseudhalenchus (FU-2)	46.81**
Mesodorylaimus (OM-5)	22.59**
Neothada (NP-2)	12.50
Prismatolaimus (BA-3)	12.50

***H* [O1]**	IndVal
Buonematidae (BA-1)	18.19**
***H* [O2]**	
Miculenchus (NP-2)	13.58
Odontopharynx (BA-1)	12.50
Alaimidae (BA-4)	25
***P* [O2]**	
Lelenchus (NP-2)	12.50
***H* [O4]**	
Hexatylus (FU-2)	50**
***H* [O5]**	
Pelodera (BA-1)	27.97**
Pelioditis (BA-1)	12.50
Seinura (PR-2)	8.20
***P* [O4]**	
Neopsilenchus (NP-2)	14.81
Tylencholaimellus (FU-4)	12.50
Mylonchulus (PR-4)	9.98
***P* [O5]**	
Paratylenchus (PA-2)	74.39**
Acrobeles (BA-2)	50.43**
Aporcelaimellus (PR-5)	28**
Aglenchus (NP-2)	12.50
Wilsonema (BA-2)	10.21

Fig. 4. – Dendrogram presenting the indicator genera assigned by IndVal to each combination of site and season. *H* and *P* before site codes stand for "harvest season" and "post-harvest season" respectively. The Indicator Value of each genus for each cluster is given, while the trophic group and c-p value are indicated in parentheses. BA: Bacterivore, FU: Fungivore, PA: Phytoparasite, NP: Non parasitic plant feeder, PR: Predator, OM: Omnivore (** P<0.01).

conventional or with the organic plots depending on the season of sampling. At a second level, older organic cultivations [O4] and [O5] were grouped together, without nevertheless masking the effect of seasonal agricultural practices on nematode communities. On the other hand, this seasonal effect was not revealed in the case of conventional plots, since [*H*C] and [*P*C] were grouped together with the lowest linkage distance.

According to IndVal method, from the total of 61 genera recorded, 19 could be considered eyrytopic, since they were found at all sites in both seasons. Among these genera no phytoparasites were found. The highest IndVal values were displayed by *Acrobeloides, Aphelenchus* and *Chiloplacus*, which were well presented at all samples constituting together about 28% of the total nematode community. It seems that the major separation of the organic from the conventional cluster was mainly due to two genera; the bacterivore *Heterocephalobus* characterized the organic cluster, while the phytoparasite *Helicotylenchus* was assigned to the conventional cluster with the highest indicator value. The second most important genus characterizing the conventional cluster was the phytoparasitic *Tylenchorhynchus*, followed by the predator *Eudorylaimus*, which however was of very low abundance. Another genus that may be considered important bioindicator, is the phytoparasite *Paratylenchus*, which was recorded only in the older organic cultivations.

Data depicted in Fig. 5, concerning the abundances of the two main indicator genera, namely *Helicotylenchus* and *Heterocephalobus,* revealed a completely opposite trend of abundance change along the gradient from conventional to recent and further to older organic cultivations.

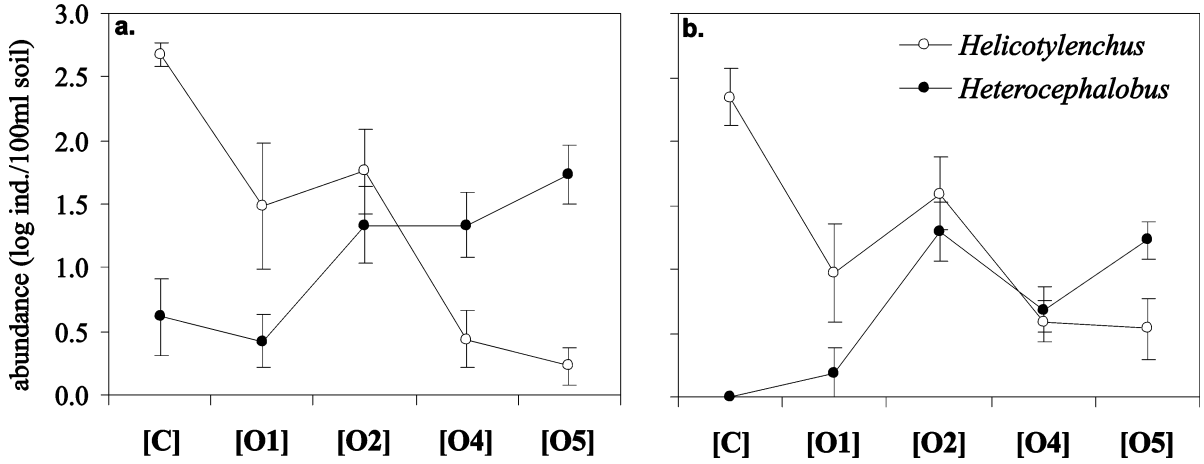


Fig. 5. – Abundance of *Helicotylenchus* and *Heterocephalobus* in the studied agricultural systems in (a) the post-harvest season and (b) the harvest season.

DISCUSSION

This study aims to demonstrate the differential effect of agricultural practices on the soil nematode community under asparagus cultivation. Our experimental design reflected two types of changes in agricultural practices. First, our plots constituted a gradient from conventional asparagus cultivation to recent organic of 1 and 2 years, which are considered transitional, and further to older and certified organic cultivations of 4 and 5 years, reflecting thus long-term effects of changing management regime. Along this gradient, changes regarding the type of nutrient input and weed control are involved. Second, the replication of samplings throughout the year reflected seasonal agricultural practices shared between both organic and conventional management.

Transition from conventional to organic

The investigation of nematode communities in soils with different history of management practices revealed several differences at various levels of community analysis. The most important finding from the analysis of trophic structure was the gradual reduction of plant feeders from conventional to the older organic cultivation, while the exactly opposite trend was revealed in the case of nematodes that feed on decomposers, i.e. bacteria and fungi. Our results support the hypothesis of VAN DIEPENINGEN et al. (2006) that the differences between the two management types are more gradual than black and white, and are in agreement with the findings of several authors (e.g. NEHER & OLSON, 1999; GARCÍA-ÁLVAREZ et al., 2004), who reported an increase of decomposer feeders and especially bacterivores under organic cultivation.

In our study, the ratio of plant feeders to decomposer feeders was 1:0.7 under conventional cultivation, changed approximately to 1:2.6 in the recent and to 1:3.8 in the older organic plots, reflecting gradual changes between the "grazing food web" and the "detritus food web" of PETERSEN & LUXTON (1982) or the "root-herbivore" and "decomposer" guilds of BRUSSAARD (1998). This shift probably resulted from the different form of nutrient input in the cultivated plots. Organic fertilizers are expected to favour bacterivores and fungivores, since they act via the microbial soil component. Feeding activity of these groups stimulates decomposition and nitrogen

mineralization (FERRIS et al., 2004). Indeed in a parallel experiment carried out in our experimental area, MONOKROUSOS et al. (2006) found that N-mineralization rates were lower in conventionally cultivated plots and increased gradually to older organic plots.

The long-term shift in trophic structure was even more pronounced when only phytoparasites instead of total plant feeders were considered. Non parasitic plant feeders actually appeared more frequently in the older organic plots as revealed by log-linear analysis and also by PPI values, since this trophic group consists of cp-2 nematodes. PPI values were significantly higher under conventional cultivation and declined gradually with increasing time of organic management, following the changes of the fraction of cp-3 parasitic nematodes. A decline of phytoparasitic nematodes in organic agro-ecosystems is reported by many authors (e.g. BOHLEN & EDWARDS, 1994; AKHTAR & MAHMOOD, 1996; WIDMER et al., 2002). MCSORLEY & GALLAHER (1995) as well as AKHTAR & MALIK (2000) have proposed that phytoparasitic nematodes may be suppressed by toxic by-products from decomposition of organic amendments and that the long-term use of the latter may even stimulate the activity of biological antagonists of nematodes. On the other hand, mineral fertilizers are known to be the reason for softer plant tissues as carbohydrates are diverted to protein synthesis instead of cell wall construction (TISDALE & NELSON, 1975), which makes plants more susceptible to phytoparasites. Thus, the gradual decrease of phytoparasites with time of organic management may indicate a log-term recovery of asparagus after ending the use of mineral fertilizers. In contrast to our results, NEHER (1999) found more parasitic nematodes in her organically cultivated plots but since the latter were cultivated by various crops, she attributed her results to host specific relationships. Moreover, FERRIS et al. (1996) claims that phytoparasitic nematodes are strongly influenced by host status of the current and previous crop. We should remind though that all experimental plots of our study were cultivated with the same perennial crop.

Apart from PPI, all other nematode indices did not change significantly with increasing time of organic management. BERKELMANS et al. (2003), who have studied nematode communities in cultivations with different history of organic management, also found that nematode indices which did not include plant feeders were insensitive to residual effects of management. Furthermore, the Maturity Index (MI) and the Structure Index (SI) indicate the successional stage of communities, which are in any case premature in cultivated soils. As NEHER & OLSON (1999) state, all agricultural practices aim at maintaining ecosystems at a more productive, pre-mature successional stage. On the other hand, both MI and SI increase with increasing contribution of predators and omnivores, which are of high c-p values, i.e. their life strategies are closer to the right end of the of the r-K continuum. However, the proportion of these trophic groups is very low in nematode communities of cultivated soils (FERRIS et al., 1996; FRECKMAN & ETTEMA, 1993; LIANG et al., 2001 among others) and in our samples they appeared sparsely.

The long-term effect of changing management regime was also obvious from the analyses at the generic level, both in terms of diversity and of community composition. Regarding diversity, our approach was based on producing diversity profiles for nematode communities instead of the commonly used diversity indices, because the latter are not fruitful in describing a multidimensional concept, such as a community, reducing it to a single number. Diversity was found to increase gradually with time of organic management. This was not due to an increase of genera richness but due to differences in dominance patterns. Under conventional cultivation, we observed strong dominance of the phytoparasite *Helicotylenchus*. A more even distribution of nematode numbers among genera was found with increasing time of organic management. The high abundance of phytoparasites and especially the strong dominance of few species among them, according to WASILEWSKA (1997), occur in long-term monocultures and are related to environmental degradation. The higher nematode diversity in the organically managed systems might also result from the increased availability of microhabitats due to organic amendments and lack of herbicides. The latter allows to an extent immigration from the weed species pool of the larger area. This is in agreement with the work of DE DEYN et al. (2004) who observed strong dominance patterns of nematodes in experimental plant monocultures and more even and diverse nematode communities in plots with higher plant diversity.

Regarding the composition of the nematode community, nineteen genera belonging mainly to the bacterivore and fungivore group were classified as eurytopic, i.e. with not specialized habitat requirements. The most abundant among them, namely *Aphelenchus*, *Acrobeloides* and *Chiloplacus* have been reported in a vast number of studies to dominate in soils under various agricultural practices, crops, ecosystems, as well as different geographic regions, climate and soil types (LIANG et al., 1999; LIANG et al., 2005a; LIANG et al., 2005b; PAPATHEODOROU et al., 2004; ILIEVA-MAKULEC, 2000; ZOLDA, 2006). Changing management regime caused gradual changes in generic structure and composition, separating the older organic plots from conventional. The relatively vague classification of recent organic plots indicates their transitional character along the gradient from conventional to organic. A striking finding was the combined decrease of *Helicotylenchus* and increase of *Heterocephalobus* with time of organic management. *Helicotylenchus* was also found in high abundance by YEATES et al. (1999) in asparagus cultivations where mineral fertilizers were applied. The bacterivore *Heterocephalobus* on the other hand was proposed by (FISCUS & NEHER, 2002) as an indicator of soil quality due to its sensitivity to disturbance, and was reported by MULDER et al. (2003) to prefer lower values of soil pH. Changes in abundance of *Heterocephalobus* might be related to the gradual pH decline from the conventional to the older organic plots of our study, which probably resulted from the long-term use of organic amendments (WIDMER et al., 2002). We should also note that the phytoparasite *Tylenchorhynchus* exhibited the same trend as *Helicotylenchus* with time of organic management. Indeed, according to VESTERGÅRD (2004) this ectoparasitic plant feeder is stimulated by N-fertilization.

Seasonal agricultural practices

Nematode indices were found useful mostly for distinguishing seasonal agricultural practices. Maturity Index (MI) was higher during October, i.e. the season with least disturbance, since fields were left with no agricultural activities for several months. The growth period of asparagus stops in autumn and during winter nutrients are translocated from the fern to the rhizome from where they will be remobilised into new spear growth in spring (FAVILLE et. al., 1999). This nutrient translocation seems to favour phytoparasites leading to a higher Plant Parasitic index (PPI) in December.

Channel Index (CI) was higher in October and December, i.e. during the post-harvest season. This indicates a shift from a more bacterial to a more fungal mediated decomposition pathway (FERRIS et al., 2001). During this period, there were amendments of plant biomass, originating either from dead weed residues after summer control or from aboveground asparagus parts, which were cut in late November and left in the fields to decompose. Plant residues constitute amendments of high C/N ratio, favouring populations of fungivores (BOHLEN & EDWARDS, 1994). Two weeks before the beginning of spear growing the soil was mounded and thus the plant residues were incorporated into the soil. Their advanced decomposition renders this organic source more labile, favouring the enrichment opportunist bacterivores with c-p value 1 (FERRIS & MATUTE, 2003). The increase of the Ba-1 functional guild resulted in significantly higher values of the Enrichment Index (EI) during March.

The distinction between the post-harvest and the harvest season was also evident from the analyses of community structure at the generic level, but mostly in the older organic plots. This was mainly due to changes in abundance of genera which were classified as eurytopic. For example *Panagrolaimus*, *Diploscapter* and *Bursilla,* which all belong to the Ba-1 functional guild, increased in the harvest season, while the Fu-2 *Aphelechoides* and *Paraphelenchus* and the root hair feeder of c-p 2 *Filenchus* displayed higher abundance in the post-harvest season. *Heterocephalobus* (Ba-2) which characterized the organic plots displayed a significantly higher abundance during this season. On the other hand, seasonal patterns were not pronounced in conventional plots. The predominance of *Helicotylenchus* (c-p 3 phytoparasite) in these plots during both seasons diminished the importance of any other changes in the contribution of the remaining species. It seems that a strong pressure as the one imposed by long-term conventional management masks the results of less intense pressures, as the ones imposed by short-term management practices.

CONCLUSION

Changing management regime of asparagus cultivation from conventional to organic as well as seasonal agricultural practices appeared to constitute driving forces altering nematode communities in different ways. More specifically, changing management regime seemed to initiate successional long-term changes in nematode communities, such as the gradual decline of phytoparasites from conventional to organic cultivation in favour of bacterivores and fungivores, the increase of non parasitic plant feeders, the gradual decrease of PPI, the increase of diversity. Alterations in the generic structure of the community were also revealed, driven mostly by the opposite trends of changes in abundance of *Helicotylenchus* vs. *Heterocephalobus*. Seasonal agricultural practices appeared to induce short-term responses of functional guilds of lower c-p values, and were reflected in all nematodes indices studied except SI. Nematode responses at the generic level to seasonal agricultural practices seemed less intense than the ones imposed by changing management regime, and in the case of conventional cultivation they were almost entirely masked.

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Pitfall trapping in flooding habitats: a new technique reveals *Archisotoma pulchella* (Collembola: Isotomidae) as new to the Belgian fauna

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ABSTRACT. Flooding habitats are unique ecosystems with complex land-water interactions. Research on their terrestrial component is seriously hindered by the lack of an adequate and efficient technique for pitfall trapping. This paper focuses on three consecutive items: (1) the description of a new type of trap, developed for use in temporarily submersed areas; (2) evaluation of its potential usefulness by a literature search of pitfall trapping in diverse environments (1998-2003); (3) its application in the trapping of arthropods inhabiting a salt marsh. The literature search demonstrates a bias towards forests, neglecting flooding biotopes. Beetles and spiders are by far the prominent taxa studied that way. Apart from some Diptera, *Archisotoma pulchella*, a new species of Collembolan for the Belgian fauna, is the only arthropod species trapped by the described sampling technique on the mud flats of the intertidal zone of the Ijzer estuary (Belgium), albeit in very high numbers. Additional sampling provided records from the Schelde estuary, and allows reconstructing some characteristics of its population dynamics.

KEY WORDS : pitfall trapping, flooding habitats, *Archisotoma*, Collembola, Belgian new species, sea shore.

INTRODUCTION

Flooding habitats, such as river banks and floodplains, temporary streams, salt marshes, creeks, inundated forests, and mangroves, are unique ecosystems with complex land-water interactions. They are inhabited by faunas of aquatic, as well as amphibious and terrestrial species, adapted to fluctuations of the water table. Terrestrial arthropods are by far the most species-rich group and play an important role in the ecosystem (DESENDER & MAELFAIT, 1999). Research on the terrestrial component of tidal marshes and other flooding habitats (DESENDER & MAELFAIT, 1999; ADIS & JUNK, 2002; BONN et al., 2002; KRUMPALOVA, 2002) is seriously hindered by the lack of an adequate and efficient technique, such as pitfall trapping, a good method of sampling because of its simplicity and ease of operation. It is an effective and cheap way of qualitatively surveying the ground surface-active arthropods over long periods of time, and allows for comparison of assemblages in different habitats (GREENSLADE, 1964). Even when it comes to diversity and gradient analysis of terrestrial arthropods in tidal marshes, sampling techniques are limited to aspirator and hand capturing with a very restricted effort of catch in time (DESENDER & MAELFAIT, 1999).

We developed a pitfall trap suitable for habitats that are regularly or unpredictably inundated. The trap is conceived in such a way that it is enclosed and protected in an air bell from the moment the rising water reaches the rim of the trap. Later on, when the water table falls back under the rim, the original situation is restored. So pitfall trapping continues as long as the trap is not submerged, regardless the time and the frequency of inundation. Other automated sampling methods are not satisfactory for two reasons: a floating pitfall trap captures only the animals on the water surface (GRAHAM et al., 2003), whereas the classic pitfall trapping method is restricted to the permanently dry portions of the habitat (MILFORD, 1999; TAJOVSKY, 1999; WISHART, 2000; IRMLER et al., 2002; DEIDUN et al., 2003; JOHNSON, 2003). This paper focuses on three consecutive items: (1) the description of a new type of trap, developed for temporarily submersed areas; (2) evaluation of its usefulness by literature search; and (3) an evaluation of its usefulness by its application in a salt march. The general design of the pitfall is described, adaptable to all types of habitats according to the specific needs of the researcher.

Archisotoma pulchella (Moniez, 1890) was lacking in the Belgian list of Collembola (JANSSENS, 2004), although it is a common species of intertidal mud flat communities in the surrounding countries (STERZYNSKA & EHRNSBERGER, 2000). Using our sampling technique, we found not only that species, but moreover we were able to reconstruct some characteristics of the population dynamics of the springtail, even when only one trap was used over a short period of time. So it is finally proved that this type of pitfall trapping yields data that generate new information, adding to knowledge of the biodiversity of flooding habitats and the ecology of their species.

MATERIALS AND METHODS

Fig. 1 presents the principle of a submersible pitfall trap before (Fig. 1A) and during (Fig. 1B) inundation, while Fig. 1C is a technical depiction of the instrument. Fig. 2 shows a photograph of the instrument with (Fig. 2A) and without (Fig. 2B) the cone, as well as the trap as

used on the study area (Fig. 2C). When the water level rises (Fig. 1Be), the pitfall (1Ba) is left dry in the air bell (Fig. 1Bf) under the cone (Fig. 1Ad). The pitfall is exposed again when the water surface drops below the rim of the container (Fig. 1Aa, Ba). Its rim has to be elevated above the substrate (Fig. 1Ag, Bg) higher than the base of the cone (Fig. 1Ad), since the air of the bell compresses when the water table rises. In our design the cone is made from a reverse plastic funnel, closed at the top with silicone glue. The difference in level between the rim of the trap and the base of the air bell inside the cone is realized by the sample holder (Fig. 1Ac), a concrete ring that fits around the trap. The instrument requires tight fixation to the substrate, to withstand uplift of the air bell during inundation. This is realized by attaching the cone (Fig. 1Cd) with rubber bands (Fig. 1Ci) to a steel support structure anchored into the substrate by a soil auger (Fig. 1Ch). This steel structure consists of three brackets on radial arms (Fig. 1Cj) welded to the top of the auger (Fig. 1Ch). A 10% formalin solution is used as a fixative (Fig. 1Ab, Cb) for the captured animals.

An exploration of the literature on pitfall trapping of animal taxa in flooding areas is undertaken for five recent years (1998-2003). The search was done on ISI Web of Science using the key words for the sampling technique 'pitfall trap' and 'pitfall trapping'. For the reference collection of habitats the key words mentioned in Fig. 3 were checked. Papers on ornithology, only dealing with avifauna, are omitted.

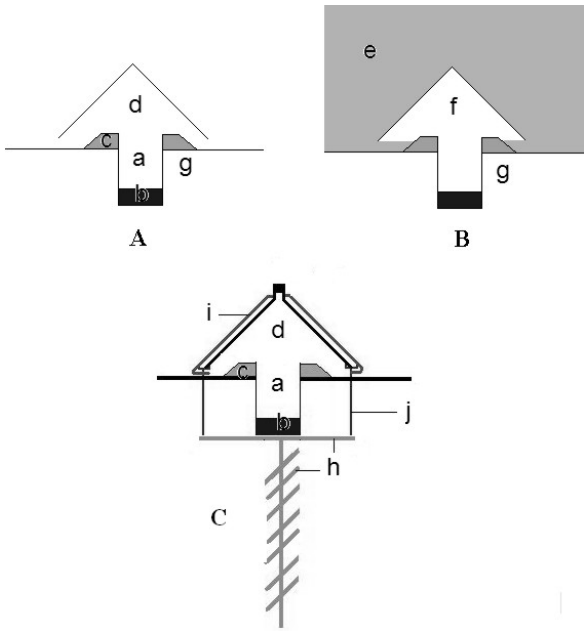

Fig. 1. – The outlines of the submersible pitfall trap before (A), during (B) the inundation, and a technical representation of the instrument (C). a: pitfall container; b: fixative; c: sample holder; d: cone (plastic funnel); e: water column; f: air bell; g: substrate; h: platform on top of the spiral; i: rubber strings; j: metallic rods.

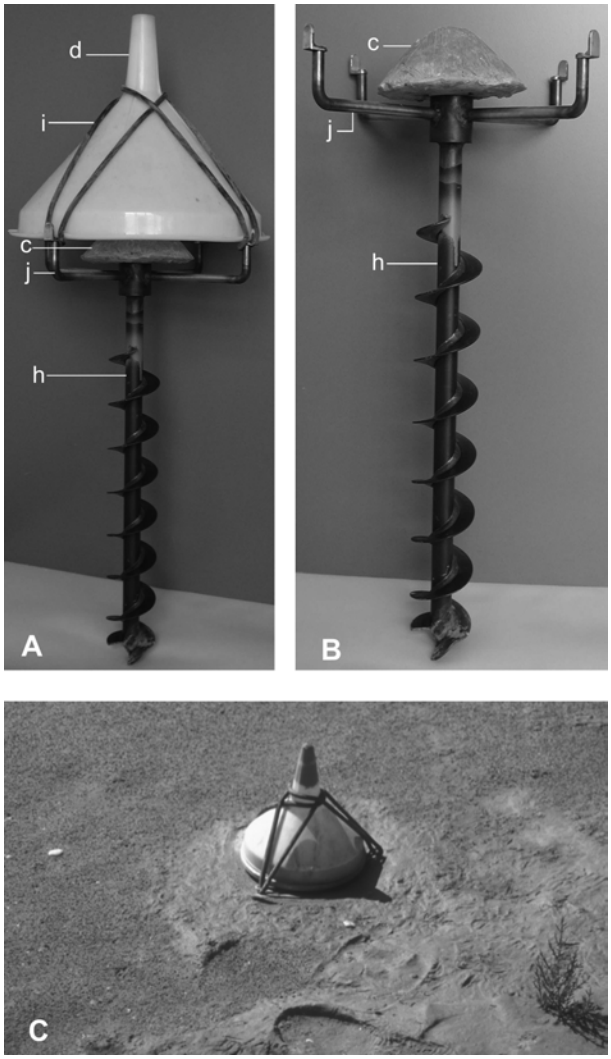

Fig. 2. – A photograph of the trap: with (A), without the cone (B), and as used on the study area (C). The numbers correspond to these of Fig 1.

The prototype of the pitfall trap was used in a salt marsh (Nature Reserve at the Estuary of the river Ijzer, Nieuwpoort, Belgium, UTM10x10: DS86) from 4 August till 15 September 2003, as shown in Fig. 2C. At intervals of two weeks the content of the pitfall was harvested (one interval corresponds to one sample), and sorted out at the lab. Apart from a few Diptera (Brachycera), the harvest was a single species collection of *Archisotoma pulchella* (Moniez, 1890). These springtails were counted and measured under a stereomicroscope (Wild M5). When a sample was too rich in specimens, a representative subsample was taken for this quantitative analysis.

Additional samples of *A. pulchella* were collected from the Estuary of the river Schelde (Kallo, UTM 10x10: ES87) at low tide on peat outcrops of the river banks, by means of a mouth-operated aspirator, on 2003 September 3, and 25 (9 and 18 specimens; legit: Frans Janssens & Jos Bruers). The taxonomic identification is based on GISIN (1960); THIBAUD & PALACIOS-VARGAS (2001); and POTAPOV (2001).

RESULTS AND DISCUSSION

The rising water surrounding the pitfall trap causes an air bell in the trap as well as under the protecting cone. Moreover, the pressure on the air bell will increase in accordance with the rising water column above the instrument. When the height between the rim of the cone and the water surface is substantial, one needs a careful evaluation of the compression of the air bell and the corresponding increase of the water level approaching the rim of the trap. Inaccurate evaluation causes flooding of the trap and loss of the sample. Those who are less mathematically inclined will arrive at acceptable results by trial and error, when simulating the trap in the appropriate environment. For whom it may concern, we could mail a detailed calculation of the physical changes when the water rises around the trap, followed by a FORTRAN code and a numerical evaluation. One has to keep in mind that the submersible pitfall is not protected against beating of waves nor strong irregular horizontal water currents. If not sheltered by one or another type of screen, each incoming wave flows over the sample holder into the pitfall, inundating the sample.

The results of the literature survey are visualised as a ranking in Fig. 3. By splitting up the references into habitat types (Fig. 3A) one observes that pitfall trapping is practised to a considerable extent in forests (35.1%) and agricultural ecosystems (31.0%); to a lesser degree in grasslands like pastures (9.3%), but only 7.2% in potentially or regularly submerged habitats. This confirms our suspicion that information based on pitfall trapping is biased towards forests and some preferred habitats, neglecting others including many types of inundated biotopes.

References of faunistic research, based on pitfall trapping, are summarised in Fig. 3B. When it comes to flooding areas, the pitfall technique is omitted in periods of risks of inundation. It has to be concluded that our apparatus opens a novel sampling methodology for pedofauna research in frequently flooded areas. Most references report on mangroves, but no information is available on their pedofauna, since the terrestrial component is limited to arboreal foraging groups, prominently ants, although arthropods on mud flats, like decapods (ASHTON et al., 2003), spiders or some insect groups (MORRISEY et al., 2003) are cited as frequently occurring inhabitants. The bulk of the papers describe the aquatic environment. Mangroves are followed by papers on floodplains and salt marshes. Here too we observe a pattern in favour of the aquatic element. If not on mammals, pedobiology focuses on beetles and spiders. Al other submersible habitats, like temporary or intermittent streams, are scarcely studied. Sampling is restricted to inundation periods (creeks) or to prolonged dry periods (temporary streams). We observe moreover that the terrestrial component of flooded areas is discriminated against, in favour of the aquatic component.

The intertidal zone of the Ijzer estuary is a single species springtail community. *Archisotoma pulchella* is the only species trapped in our pitfall on the clay flats of the salt marsh, but in very high numbers. This species belongs to the family Isotomidae (order Entomobryomorpha, class Collembola; Syn.: *Isotoma pulchella* Moniez, 1890). It is a new species for the Belgian fauna.

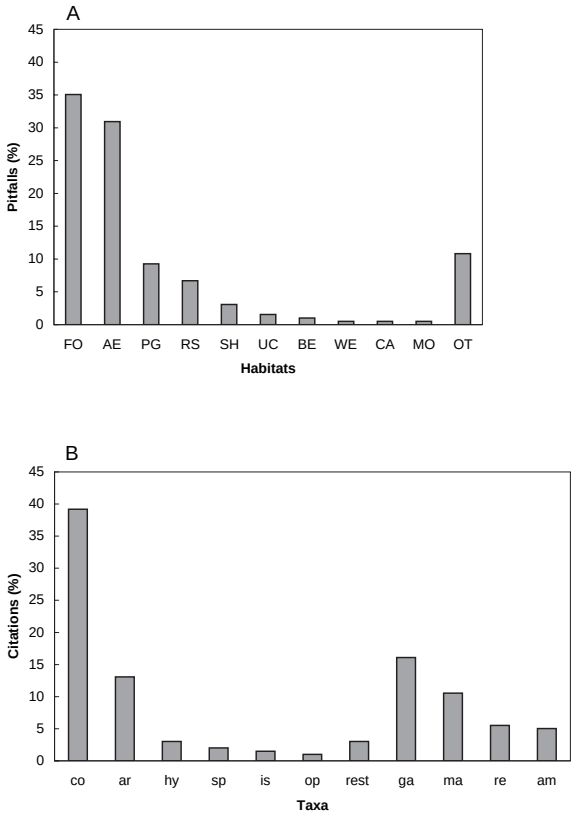

Fig. 3. – The results of a literature search of pitfall trapping in different habitats (A), and of the animal taxa collected in flooding areas (B) for five recent years. Habitat classes (key words): FO: forests, AE: agricultural ecosystems, PG: pastures, RS: riparian habitats, SH: shrub habitats, UC: urban areas, cities, BE: beaches, WE: wetlands (including marshes, fen and bog habitats), CA: caves, MO: moor lands, fell fields, grassland, river banks, shore lines of lakes, OT: others (glacier foreland, open ruins, desert, and/or undefined habitats).-The taxa are: co: Coleoptera (beetles), ar: Araneae (spiders), hy: Hymenoptera (ants), sp: Collembola (springtails), is: Isopoda, op: Opiliones, rest (Acari, Annelida, Aves, Bacteria, Chironomidae, Copepoda, Decapoda, Diplopoda, Ephemeroptera, Hemiptera Isopoda, Mollusca, Myriapoda, Nematoda, Odonata, Orthoptera, Pisces, Plecoptera, Porifera, Rotatoria, Trichoptera), ga: arthropods (general), ma: Mammalia, re: Reptilia, am: Amphibia.

The species is known from the coasts of France, Germany, The Netherlands and England. When it occurs at the sea shores of all these surrounding countries, it is evident that the Belgian gap is filled now. Known as a moderately common, widespread, littoral species, it prefers muddy or sandy surfaces on low sea shores. As a stenohaline species, it is abundant on the water surface of saline pools and under stones. The distribution pattern of *Archisotoma* communities in the soils of intertidal salt marshes is described by STERZYNSKA & EHRNSBERGER (2000). These authors found that the springtails colonize intertidal 'mud' soils to a depth of 30cm and at a distance of 10m towards the sea from the coastal edge. Our results

confirm these data. Gut contents show transparent empty shells of digested diatoms (Fig. 5). Using our sampling technique, we found not only the species, but moreover we are able to reconstruct some characteristics of the population dynamics of the springtail, even though only one trap was used over a short period of time (Table 2 and Fig. 4). The numbers increase during the sampling campaign, starting from the transit of the dry hot summer period (4th of August), arriving at the high numbers in the second half of August, to a maximum at the end of the sampling period in October. In contrast, *Archisotoma* was not trapped in pitfalls at the nearby flood line, nor were common species among the amphipods, spiders, beetles and other taxa of the pitfalls on the flood line of the same sampling site found in the submersible pitfall (HOFFMANN et al., 2004). Only some individuals among the Diptera were trapped in the submersible pitfall, belonging to the families Anthomyidae (6 specimens), Chloropidae (1), Dolichopodidae (23), Ephydridae (7), Sphaeroceridae (2), and one Staphylinid beetle. Spiders and isopods were not captured by this type of trap. One could conclude that they don't walk on such mud flats.

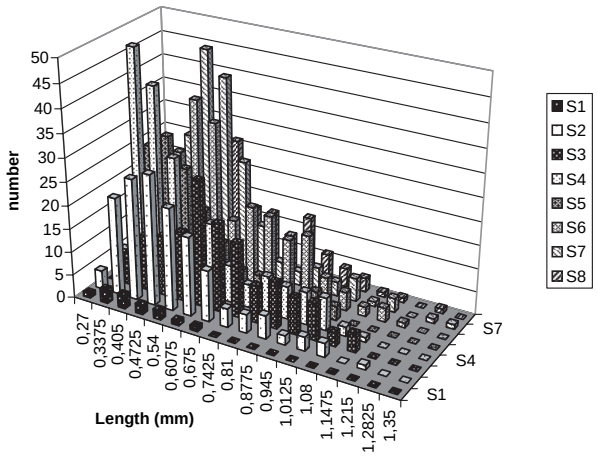

Fig. 4. – Distribution (numbers) of body length (mm) for the different sampling periods, arranged in time according to Table 2.

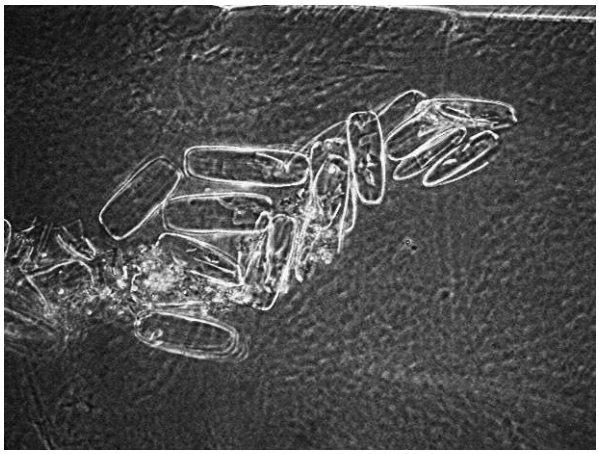

Fig. 5. – The gut content of *Archisotoma pulchella*, showing digested empty shells of diatoms.

TABLE 1

The literature search (1998-2003) on animals in submersible habitats, subdivided according to their components (water column, benthos, pedofauna and arborel); the numbers correspond to the amount of records (between brackets all cited taxa; the abbreviations are: ac: Acari, an: Annelida, ar: Araneae, av: Aves, ba: Bacteria, ch: Chironomidae, co: Coleoptera, cp: Copepoda, de: Decapoda, ep: Ephemeroptera, ga: general, arthropods, he: Hemiptera, hy: Hymenoptera, ma: Mammalia, mo: Mollusca, ne: Nematoda, od: Odonata, pi: Pisces, pl: Plecoptera, po: Porifera, re: Reptilia, ro: Rotatoria, tr: Trichoptera, un: unicellular.

aquatic		terrestrial	
mangrove: 40			
water column: 13 (pi 10; he 1; cp 1)	benthos: 23 (mo 5; de 5; ga 5; an 4; ne 2; ba 2; ac 1; po 1; un 1)	pedofauna: 0	arboreal: 7 (hy 4, ac 1, re 1, ga 1)
floodplain: 36			
water column: 18 (pi 8; ga 5; ro 2; cp 1; od 1)	benthos: 9 (ch 5; an 2; ga 2; mo 1; pl 1; ep 1)	pedofauna: 7 (ma 3; co 2; ar 2; mo 2; ac 1)	arboreal: 0
salt marsh: 27			
water column: 14 (pi 10; de 4; ga 3; cp 1; un 1)	benthos: 7 (ga 6; de 3; ne 2; an 2; mo 2; av 1)	pedofauna: 1 (co 1; ar 1)	arboreal: 0
temporary / desert / intermittent stream: 10			
water column: 0	benthos: 7 (ga 6; pl 1)	pedofauna: 2 (ga 2)	arboreal: 1 (tr 1)
creek: 6			
water column: 2 (pi 2)	benthos: 4 (ga 3; mo 1)	pedofauna: 0	arboreal: 0

TABLE 2

Number and capture rate (springtails/day) of *Archisotoma* collected in the submersible trap over a continuous sampling period, divided in 8 intervals (sampling time: day/month).

sampling time	04/08	12/08	18/08	25/08	01/09	08/09	15/09	06/10
number	2	810	2610	1700	4680	2640	1290	5540
capture rate	0.3	101.3	435.0	242.9	668.6	377.1	184.3	26.3

CONCLUSION

Although pitfall trapping is a widely used technique for studying pedofauna communities, this sampling method is not used for habitats subjected to flooding, due to the lack of a well adapted trap. Here we present a device enveloping the pitfall trap in an air bell during the whole period of inundation, leaving the trap functional when the water retreats. This type of sampling generates new information, adding to the knowledge on biodiversity and ecology of terrestrial wetland species, as we proved for the springtail *Archisotoma pulchella*.

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Multiyear homing and fidelity to residence areas by individual barbel (*Barbus barbus*)

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ABSTRACT. Nine barbels (*Barbus barbus*) from the River Ourthe (River Meuse basin) were equipped with transmitters programmed to switch ON during two consecutive spawning seasons in 1998 and 1999 (April to July). Six of the nine barbels tracked in 1998 were also tracked in 1999 during the same period. The length of the spawning migration ranged from 200 to 22700m. After the spawning activity observed from 12–16 May 1998 and 4–6 May 1999, the barbels homed to the site occupied before spawning. Each barbel used the same spawning area in 1998 and 1999, despite the presence of other spawning sites on their migratory route. These observations revealed the existence of strict reproductive homing in the barbel and a long-term fidelity to particular resting places.

KEY WORDS : migration, homing, reproduction, *Barbus barbus*, telemetry.

Fidélité inter annuelle aux sites de pontes et aux aires de résidences chez le barbeau fluviatile (*Barbus barbus*)

RÉSUMÉ. Neuf barbeaux fluviatiles de l'Ourthe (bassin de la Meuse) ont été équipés d'émetteurs radio programmés pour fonctionner durant deux saisons de reproduction consécutives (avril-juillet 1998 et 1999). Six des neufs poissons suivis en 1998 ont été retrouvés et suivis pendant la même période en 1999. L'ampleur des migrations de reproduction, unidirectionnelles vers l'amont, a varié de 200 à 22700m. Après le frai (12-16 mai 1998; 4-6 mai 1999), le retour direct et précis vers le gîte occupé avant la migration a été observé chez tous les individus. En 1999, aucun poisson n'a utilisé une frayère différente de celle fréquentée en 1998, et ce malgré la présence éventuelle d'autres sites de ponte sur son trajet migratoire. Ces observations traduisent l'existence d'un homing reproducteur assez strict chez les individus de *Barbus barbus* ainsi que d'une fidélité à long terme vis-à-vis d'un gîte de résidence particulier.

INTRODUCTION

The study of spawning homing in fish has long interested researchers and has been studied most in the different anadromous salmonid species (STABELL, 1984). Using marking-recapture techniques (YOUNGSON et al., 1994), experiments that moved migrating spawners (O'CONNOR & POWER, 1973) or released juveniles raised in hatcheries (POWER & MCCLEAVE, 1980; PASCUAL et al., 1995) have demonstrated that most species of anadromous salmonids have a general fidelity to the birth river (PAPI, 1992; QUINN, 1993). This interest is warranted as much by the mystery surrounding this animal capability and the biological mechanisms involved as by the socioeconomic stakes at play in the intensive farming of migrating salmonid populations.

In freshwater-resident, non-anadromous fish species, interannual fidelity to a precise spawning area has also been observed. In these species, spawning activities are regularly observed on the same spawning sites from one year to the next (*Esox Masquinongy:* CROSSMAN, 1990; *Leuciscus leuciscus:* CLOUGH & LADLE, 1997; *Salvelinus alpinus*: FROST, 1962; *Salmo trutta*: OVIDIO, 1999; *Thymallus thymallus*: OVIDIO et al., 2004; PAVLOV et al., 1998; *Rutilus rutilus:* GOLDSPINK, 1977; L'ABBÉE-LUND &VOLLESTAD, 1985). Demonstrating this demecological characteristic has led to improvements in protective measures and restoration of spawning grounds and has contributed additional arguments in favour of maintaining free movements for fish in streams.

However, fidelity to a spawning site over several successive spawning seasons has rarely been observed at the individual scale. In Placentia Bay (Newfoundland, Canada) ROBICHAUD & ROSE (2001) observed that certain Atlantic cod, *Gadus morhua*, individuals used the same spawning site from one year to another. In freshwater species, similar observations are lacking in the scientific literature. Studying this fidelity in terms of spawning

ground requires tracking an individual over at least 2 years. This methodological requirement is difficult to satisfy using passive individual marking techniques. Yet recent technological progress in the field of aquatic telemetry has improved transmitters to extend their lifetime and to equip them with an internal clock to program the transmission period (duty cycle transmitters). This technical sophistication considerably expands the experimental range, notably in terms of the restrictions imposed by the transmitter's limited lifetime, particularly when the fish to study are low in weight. This type of study of individual behaviours over a protracted part of the life cycle can provide information on the fishes' life histories and behavioural choices, thus improving our understanding of population biology and the evolutionary consequences of life cycle modifications.

Long migrations towards spawning sites have been observed in different fish species residing in rivers. The barbel, which in Western Europe is often a good part of the fish biomass in medium-sized and large gravel bed streams, shows this migratory behaviour during the spawning period (BARAS, 1992). In the River Ourthe, a tributary of the Belgian River Meuse, which shelters the barbel population studied here, many spawners gather every spring on a few spawning grounds distributed along the river (BARAS, 1992). Observation of this behaviour has naturally raised the question of a possible interannual fidelity of the individual to a spawning site, a hypothesis that has been tested in the present study in the barbel, a good study model because of its longevity and because it is highly representative in the river studied.

MATERIAL AND METHODS

Study site

The study took place in the River Ourthe, the main tributary of the River Meuse in Belgium (Fig. 1, in a 27km long stretch between the Chanxhe dam downstream and the Barvaux-sur-Ourthe dam upstream. At this spot, the river's slope is a mean of 1.5‰, for a width between 25 and 30m at the low water level and a mean annual flow rate of 22.9m^{3}s^{-1}. The water temperature was studied in a continuous manner using a thermograph (Richards Instrument, precision 0.1°C) situated at Hamoir-sur-Ourthe (50°25'36'' N, 5°32'25'' E). From 1989 to 1999, the water temperature varied from 0 to 26.8°C. The flow rate of the River Ourthe was recorded every hour at Durbuy (data from the D.G.R.N.E. Water Division). The ichthyofauna of the sector studied was dominated for the most part by *Barbus barbus* (up to 50% of the biomass observed; PHILIPPART, 1987; BARAS, 1992), mostly associated with *Rutilus rutilus* (L.), *Thymallus thymallus* (L.), *Salmo trutta* (L.), *Leuciscus cephalus* (L.), *L. leuciscus* (L.), *Chondrostoma nasus* (L.) and *Barbatula barbatula* (L.).

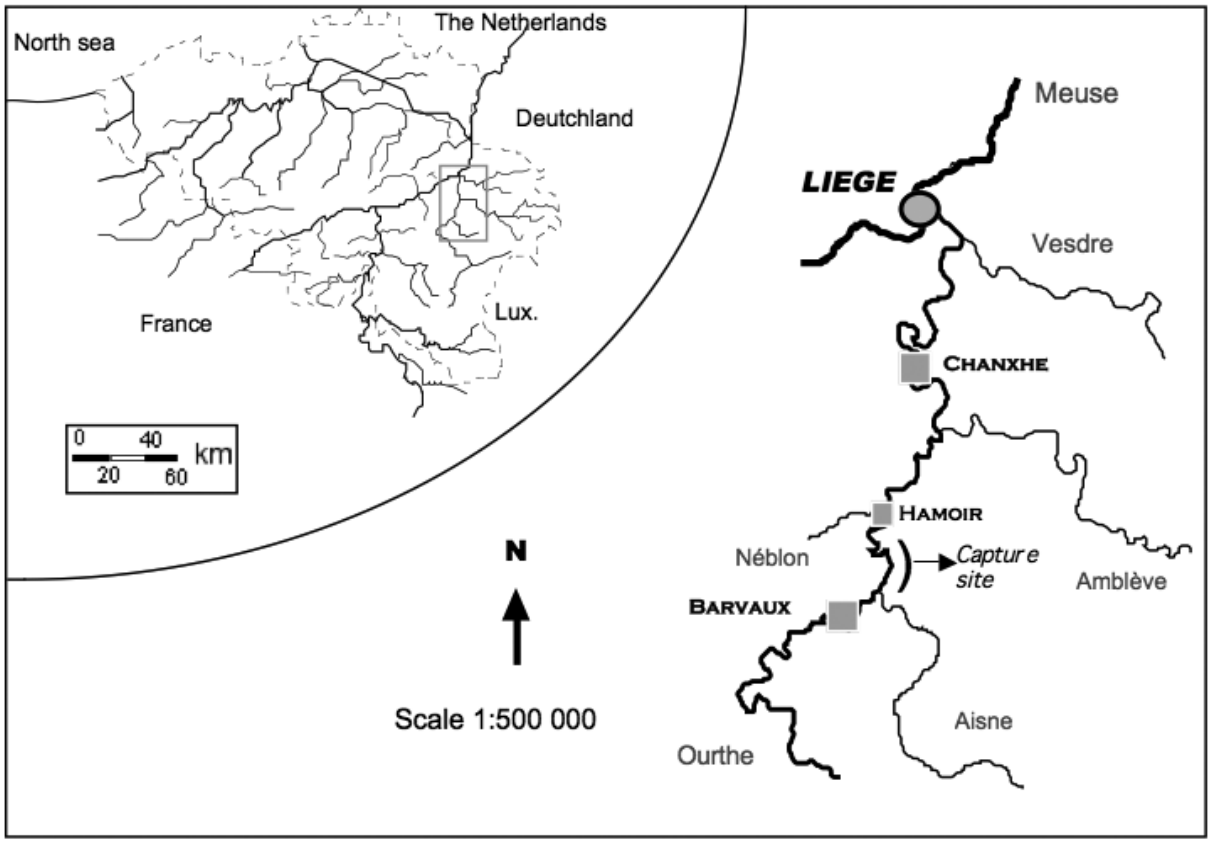

Fig. 1. – Localization of the study site in Belgium, between Barvaux and Chanxhe on the main reach of the River Ourthe.

TABLE 1

Characteristics of the barbels radiotracked in the Ourthe at the time they were captured in April–May 1998.

Fish	FL (mm)	Weight (g)	Sex	Date de capture	Transmitter weight (g)	Tag ratio* (%)
1	462	1172	Female	6 May 1998	18.5	1.58
2	429	957	Female	6 May 1998	18.4	1.92
3	427	935	Female	6 May 1998	18.5	1.98
4	430	961	Female	6 May 1998	18.8	1.96
5	420	927	Male	6 May 1998	18.4	1.98
6	481	1404	Female	5 May 1998	18.5	1.32
7	438	944	Female	6 May 1998	18.8	1.99
8	428	1063	Female	5 May 1998	18.5	1.74
9	415	874	Female	28 April 1998	18.6	2.12

* ratio between the weight of the transmitters and the weight of the fish

METHODS

Nine barbels (B1–B9; Table 1) were captured using electric fishing (Deka 5000), from 28 April to 6 May 1998, in the sector between Hamoir and the confluence of the Ourthe with the Aisne. The fish were anaesthetised with 2-phenoxyethanol (0.4mL l–1) and a radiotransmitter (40MHz, internal antenna, 18.5g in the air, 68×16mm in diameter, ATS, Inc.) was inserted in the intraperitoneal cavity through a midventral incision between the anogenital papilla and the insertion of the pelvic fins. The incision was closed with three suture stitches (resorbable 3/0 catgut on a 16mm needle). The transmitters used were programmed to only function (emit a signal) during a period of 70 days between 28 April and 7 July, and to remain inactive during the 295 days completing the annual cycle, with this procedure repeated until the transmitter's battery ran out, which allowed us to follow six of the nine barbels over two consecutive spawning seasons (1998–1999). After tagging, the fish were released at their capture site, immediately after they recuperated their swimming and orientation capacities. The transmitter to marked fish weight ratio remained less than 2% (except for barbel no. 9: 2.12%; Table 1), which is considered a very comfortable mass ratio (WINTER, 1996; JEPSEN et al., 2002).

The fish were localized during the day (diamond directional antenna, Fieldmaster receiver, ATS, inc.). In 1998, fish were localized every day from 28 April to 22 May and every 2 days from 23 May to 5 July. From 28 April to 6 July 1999, the fish were positioned three times a week. Movements were calculated with a precision of 10–15m based on field markers or using topographic maps. The main spawning sites (whether or not they were used by the barbels tracked in 1998 and 1999) were identified along the entire sector by observing spawner gatherings while walking along the study sector during the spawning period. Some of these spawning grounds had already been identified during earlier studies by PHILIPPART (1987) and BARAS (1992).

Barbel mobility was characterized by indicators at different spatial scales, defined below (BARAS, 1992):

– Net longitudinal movements: an indicator of spatial mobility corresponding to the distance separating two locations;

– Longitudinal home range: the area occupied by an individual where it developed all its activities. It is expressed by its longitudinal extension determined by the distance between the most upstream location and the most downstream location. For a single individual, it can be calculated at different time scales (daily, monthly, seasonal, annual) and can therefore contain (depending on the time scale chosen) the spawning area.

– Residence area: a reduced-surface zone in which the barbel is localized most frequently, outside of the spawning zone. It can develop one activity (feeding) in this zone or rest.

RESULTS

Six barbels out of nine marked in 1998 were also tracked in 1999 (Fig. 2). Barbels B3 and B5 were caught by line fishermen in July–August 1998 and the transmitters were returned. Fish B4 was found dead, much thinner, in the Ourthe on 4 May 1999, 10200m downstream from its last localization (6 July 1998).

Spawning migrations

In view of the divergence between the initial capture site and the localization after the post-spawning downstream migration, six of the nine barbels were marked, in 1998, probably during their migration towards the spawning sites. Consequently, it was impossible to date the start of migration, except for B4 and B8: 16 May and 11 May 1998, respectively. In 1999, the start of migration took place between 4 and 6 May, except for fish B2, which participated in no spawning migration after its transmitter was started up, and for B6, which was found on 28 April 1999 downstream of the Barvaux dam, near its spawning site, and which had therefore done its spawning migration before the transmitters were started up.

For 2 years, the migrations were unidirectional, from down- to upstream for all fish except B2 and B7, which in 1998 spawned 2500m downstream from the capture site (Fig. 2). The distance separating the upstream limit of the spawning migration from the resting place after downstream migration was between 250 and 22700m in 1998 and between 200 and 6780m in 1999 (Table 2). In view of the occupation of the same resting place by fish before (1999) and after (1998–99) the spawning period, these

values correspond to the total migration distances covered during the 2 years of tracking. The absence of migrations greater than 10000m in 1999 stems from the fact that the two fish that covered these long migration distances in 1998 (B3 and B4) could not be tracked or did not migrate at all in 1999 (B2). Movements were always fast, because the fish were localized near the spawning grounds less than 48h after the start of migration.

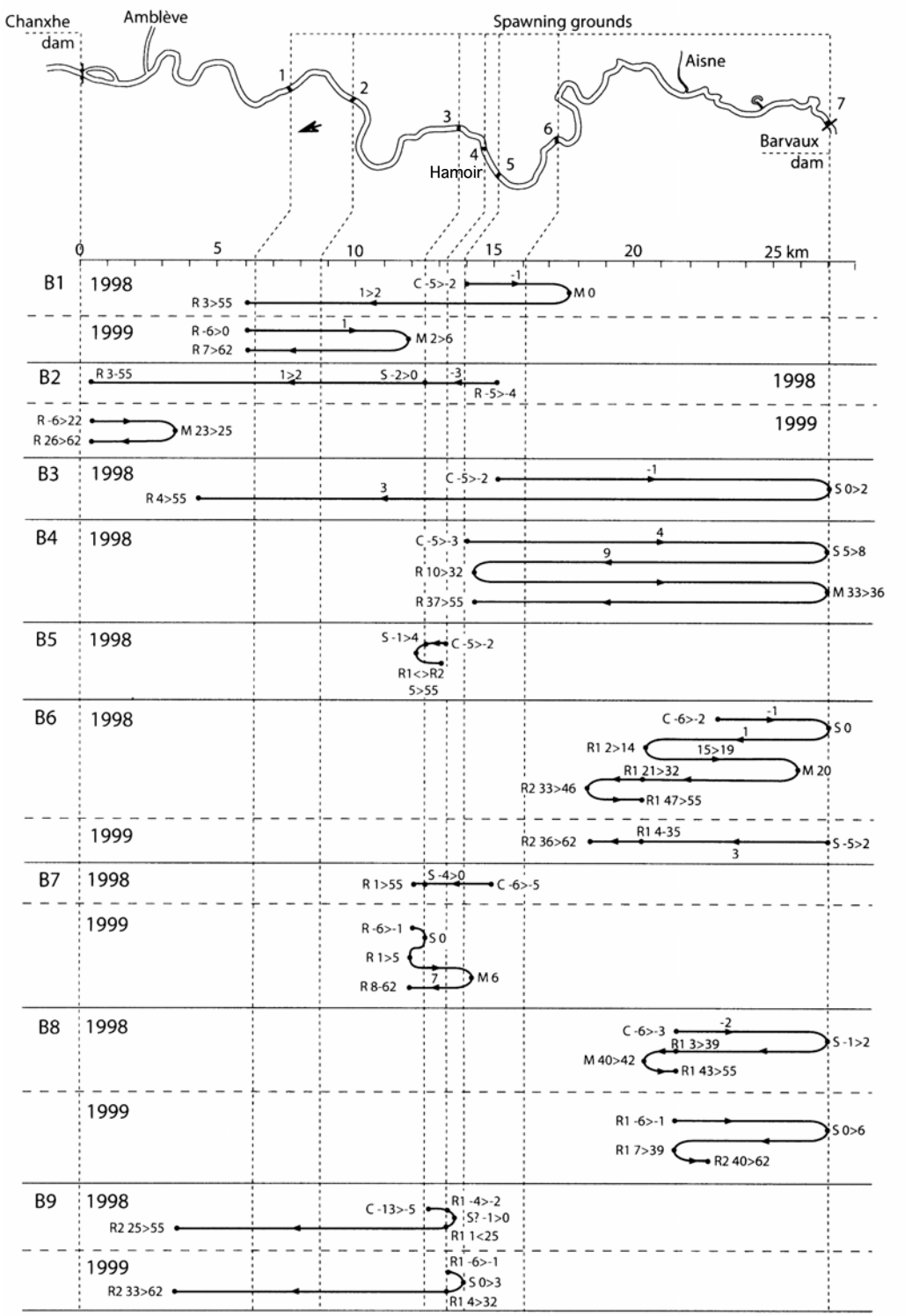

Fig. 2. – Mobility of the radiotracked barbels in the River Ourthe in 1998 and 1999. The study zone is situated between the Chanxhe and Barvaux dams. The different spawning beds identified were numbered 1–7, downstream to upstream. Individual movements are represented on continuous lines along a distance scale, with mention of the capture site localization (C), the residence area (R), the spawning bed (S) and the upstream limit of migration with no spawning bed observed (M). The time spent by the fish at the different sites and the duration of its migration are indicated on the graphs. Day 0 corresponds to the date of the first episodes of spawning observed in the population for the year considered.

TABLE 2

Mobility characteristics of the barbels radiotracked in the Ourthe in 1998 and 1999. The statistics include nine fish in 1998 and six in 1999 (see the text for details).

	1998	1999
Post spawning home range* (m)	Range: 340-12855	Range: 280-10100
Length of spawning migration (m)	Range: 250-22700	Range: 200-6780
Date of migration to the spawning ground*	9 to 16 May	< 28 April to 6 May
Date of post spawning homing	14 to 22 May	6 to 11 May

* after the tagging in 1998

Activity on spawning sites

In both 1998 and 1999, spawning began after a substantial rise in the temperature. In 1998, spawning was observed from 12 to 16 May, for a minimal daily water temperature between 16.6 and 18.0°C, and a flow rate ranging from 9.8 to 12.04 m^3s^{-1} cm. In 1999, spawning spread over the period from 4 to 6 May with a minimal daily water temperature between 13.1 and 13.2°C and a flow rate from 10.13 to 10.66 m^3s^{-1}, with activity starting up again on the spawning sites on 11 May. The radiotracked fish spent between 1 and 8 days in the spawning area (less than 100m upstream or downstream). Nevertheless, the time spent on the spawning site itself did not extend beyond 1 day, except for fish B5 (4 consecutive days).

TABLE 3

Localization of the spawning sites identified in the study zone and use by the radiotracked barbels.

Spawning site		1	2	3	4	5	6	7
Localization* (m)		6375	8750	12500	13300	13975	16100	26625
Used by tagged barbels n°	*1998*	–	–	B2-5-7	–	B9(?)	–	B3-4-6-8
	1999	–	–	B7	–	B9	–	B6-8
Swam past by tagged barbels n°	*1998*	–	–	–	B2-7	B2-7	B1-3-4	–
	1999	B1	B1	–	–	–	B4	–

* distance from the downstream limit of the study area (barrage de Chanxhe)

Interannual fidelity to the spawning site

Seven spawning beds used by the barbels every year were observed from 1989 to 1999 in the sector studied (Table 3). These sites were made up of large central deposits of gravel (sites 1, 3, 5 and 7) or by lateral convex banks covering a smaller area (sites 2, 4 and 6), three of which (3, 5 and 7) were used by the radiotracked barbels. The fish did not spawn systematically at the site nearest to the resting place occupied before the migration start-up. Indeed, the spawning route of several barbels (five in 1998 and two in 1999) included at least one indexed spawning site.

Of the six barbels tracked in 1998 and 1999, three (B6, B7 and B8) were localized at the same spawning site both years (Fig. 2; Table 3). Fish B9 was localized at spawning bed no. 5 on 4 May 1999 and 400m downstream on 12 May 1998. However, it cannot be systematically excluded that it spawned at this site in 1998. Fish B2 visited spawning site no. 3 in 1998, but undertook no spawning migration in 1999. Finally, fish B1 could not be localized near any spawning bed in 1998, even though it migrated 12300m upstream. In 1999, it stayed 3 days (6–10 May) 500m downstream from spawning site no. 5. None of the barbels was localized in 1999 on a spawning site different from the one used in 1998.

Post-spawning homing, interannual fidelity to the summer resting place

The six barbels tracked for the 2 years all manifested precise post-spawning homing in 1999 and fidelity from one year to the next to the resting place occupied during the pre- and post-spawning period. In 1999 all fish, with the exception of B6, which had already done its spawning migration, were found in their respective resting places they had occupied after the downstream migration in 1998 (Fig. 2). They were localized again in the same places after the downstream migration in 1999. Returning to the resting place within 48h was observed for all fish between 14 and 22 May in 1998 and between 6 and 11 May in 1999.

Mobility outside the spawning period

Excluding spawning migrations, the home range of the fish tracked was between 340m and 12855m in 1998 and between 280m and 10100m in 1999 (Fig. 2). Six of the nine radiotracked barbels only occupied a single resting place regularly during the tracking period (B1, B2, B3, B4, B7 and B8). In 1998, two barbels left the resting place occupied after the downstream migration that followed spawning and migrated towards a secondary resting place, situated downstream from the first. This behaviour was repeated during the same period in 1999. Fish B6 left its resting place (between 13 June 1998 and 9 June 1999),

situated in a deep calm, and stabilized 2210m downstream in a similar habitat. Similarly, fish B9 moved from one resting place to another 10060m downstream (7 June 1998 and 7 June 1999). Fish B5 was localized alternately (1998) in a resting place near spawning site no. 3 and another situated 700m upstream of the first.

Other distant movements were observed outside of the spawning migrations. Amongst the most remarkable, let us cite the movements made by fish B4 (12675m upstream, from 14 to 17 June 1998, returning to the resting place on 18 June) and B6 (5650m upstream, from 27 May to 1 June 1999, returning to the resting place on 2 June 1999).

DISCUSSION

This study is original in that it provides individual daily tracking of six female barbel spawners over two consecutive spawning seasons. Radiotelemetry techniques, in particular duty cycle transmitters, allowed us to target a precise period in the annual life cycle of the species and to track the fish for more than 1 year. It cannot immediately be excluded that a behavioural disturbance was not induced by implanting the transmitter and the fish carrying it. However, the methodology implemented here was identical to that which had been used in previous studies on *Barbus barbus* (BARAS, 1992, 1995; LUCAS & FREAR, 1997), which showed that the fish was highly tolerant to the transmitter implantation operation. In addition, the low values of the transmitter weight to fish weight ratio (1.32–2.12%; Table 1) can reasonably exclude that there was significant alteration in its swimming capacity.

Great variability was found to characterize the respective mobility of the different barbels tracked during the spawning period. The distance separating the residence area occupied before spawning migration from the spawning site varied from 250 to 22700m. These observations are in agreement with observations of barbels on the River Severn (England) by HUNT & JONES (1974), and the River Jihlava (Czech Republic) by PENÁZ et al. (2002), who distinguished two fractions, mobile and static, within a single population. At the end of migration, the spawners grouped on spawning sites, relatively few in number considering the dispersion of individuals before spawning. Spawning activity was concentrated on only a few days (4 days in 1998; 3 days in 1999). Several hundred individuals can be observed simultaneously on the same spawning bed. This synchronization of spawners can be explained by considering the factors setting off spawning and the demands of the species in terms of spawning habitat, which are now well known. BARAS & PHILIPPART (1999) have clearly shown that the arrival of barbels on the spawning grounds of the River Ourthe and spawning start-up respond to an increase in water temperature (minimum daily T>13.5°C). Grouping of a large number of spawners at the spawning sites may result from their precise requirements in terms of spawning microhabitat (BARAS, 1994). This precise environmental control of the spawning activity tends to maximize the spawning success of the species by ensuring the embryos have a thermal environment favourable to their survival and their rapid development (BARAS & PHILIPPART, 1999).

The individual's attachment behaviour to a particular habitat was manifested in three ways in the barbels tracked during this study: (i) fidelity to the spawning site, (ii) post-spawning homing, which was observed by OVIDIO (1999) in the brown trout (*Salmo trutta*), and (iii) fidelity to a precise residence area, from one year to another. These observations, conducted on a small number of female individuals over a 2-year period, should, however, be interpreted cautiously, as a preliminary approach to the issue of *Barbus barbus* homing.

It is difficult to conclude that the use of the same spawning ground over 2 consecutive years, as was observed in three fish out of six resulted from a limited availability of favourable habitat that brought these fish together on the same site every year. Indeed, the spawning route of several barbels included active spawning grounds that were not visited. The expression of this homing behaviour as it was observed in the barbels tracked implies the existence of a mechanism by which this fidelity is acquired, as well as the development of precise sensory recognition of the site involved (BRAITHWAITE & BURT DE PERERA, 2006). The hypothesis of an early olfactive impregnation mechanism (between the emergence from gravel and smolt downstream migration) enjoys general agreement in terms of birth river fidelity on the part of the different anadromous salmonid species (GROVES et al., 1968; STABELL, 1984; QUINN & DITTMAN, 1990). This mechanism can explain the spawner's migratory orientation towards the birth river, through recognition and discrimination of olfactive landmarks that it has been exposed to and is sensitive to at the beginning of its life cycle. Transposing this theory to species such as the barbel whose movements are limited to a single stream implies the presence of olfactive markers specific to a precise site, and no longer to a river or a river reach. The attraction of spawners by other individuals already present at the spawning ground or the upwelling of ground water recognized by the fish (AUDET et al., 1985) have been suggested in this context. In addition, if the movements take place in a familiar and spatially restricted environment, the fish probably use visual landmarks for orientation (BRAITHWAITE & BURT DE PERERA, 2006). In this case, acquisition of fidelity to a precise site and learning the migratory routes suggests a social transmission of the information, with young individuals following the older ones during migrations (DODSON, 1988). This learning mode has clearly been demonstrated in certain ocean reef fish (Haemulidae; HELFMAN & SCHULTZ, 1984) and has been proposed by OLSON et al. (1978) to explain the spawning migrations as well as how the spawning sites were selected in walleye (*Sander vitreus*). This mode of acquisition and manifestation of fidelity to the spawning site (and to resting place(s)) is highly plausible in the barbel given its shoaling behaviour and the great spatial precision of post-spawning homing.

The adaptive value of spawner homing in *Barbus barbus* can undoubtedly be explained by the same arguments: spawner grouping and matching of the spawning microhabitat selected with the ecological demands of the embryos during the subgravel stage of life. This philopatric behaviour should also influence the mixing of genes within the population. If this were confirmed by other studies, it would also indicate a certain genetic isolation

of spawning zones despite their geographic proximity. However, this isolation cannot be expected to be strict and definitive, since the river dynamics of a stream can make spawning sites disappear or appear over the years. This type of phenomenon would inevitably cause an individual behavioural adaptation of the spawners that would be interesting to study.

In terms of river management, several recommendations can be made for these populations. Spawning beds (often targeted by riverbed cleaning works) should be maintained and protected, even if replacement sites seem available nearby or are constructed artificially. Given the great distances certain individuals travel, access to these spawning beds can only be guaranteed if fish can circulate freely on extended river stretches (several dozen kilometres). Dams whose construction cannot be avoided should be equipped with fish ladders that are effective and that can be used by different families of fish whose swimming and leaping capacities are sometimes quite different (Ovidio & Philippart, 2002; Ovidio et al., 2007).

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Effect of prey- and predator size on the capture success of an aquatic snake

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ABSTRACT. Large aquatic snakes typically do not include small prey into their diet. This is hypothesized to be so because small preys are difficult to catch in an aquatic environment. Also an effect of snake size on capture success is plausible, with large snakes having a lower capture success then small snakes for similarly sized prey. We tested the effect of snake- and prey-size on the capture success of the specialized aquatic snake *Natrix tesselata*. No effect of snake size on capture success was found for the size range that was tested. Possibly size becomes only important from a minimum absolute size (larger than the maximal size tested in our study) onwards. Unexpectedly, *Natrix tesselata* needs fewer attempts to capture small fish. In contrast, *N. maura*, a congeneric more generalist species, needs fewer attempts to capture larger fish. A possible explanation for this conundrum lies in difference in the degree of specialization between these two species. An in-depth study of the hydrodynamics of this snake-prey system could provide ways to evaluate the importance of size effects.

KEY WORDS : capture, *Natrix tesselata*, prey size, specialisation

INTRODUCTION

Whereas the diet of most large terrestrial snakes contains both large and small prey (ontogenetic telescope in lower prey size limit), the amount of small prey in the diet of aquatic snakes typically decreases when they grow larger (ontogenetic shift in lower prey size limit, see ARNOLD, 1993; Table 1). Thus, large aquatic snakes appear to no longer include small prey in their diet. Previous authors have posited two non-mutually exclusive hypotheses to explain this phenomenon.

First, absolute snake size in itself might affect capture success. Due to the density and viscosity of water, large snakes are predicted to push more water away in front of their heads compared to small snakes (proportional to the surface area exposed to the flow; see VOGEL, 1994). With respect to feeding, this means that a large snake could potentially push the prey away from, or to the side of its own head, or alarm the prey earlier by triggering its lateral line system through the displaced water column. Both these effects could then result in a decreased capture success of large snakes (for more background on the dynamics of prey capture under water in vertebrates see e.g. MULLER & OSSE, 1984; VAN LEEUWEN, 1984; LAUDER, 1985; YOUNG, 1991; VOGEL, 1994). Thus, due to the physical properties of the aquatic environment large snakes are predicted to have lower capture success then small snakes.

Second, small prey might simply be difficult to capture in aquatic environments, independent of snake size. As large fish have larger surface areas, they will resist potential bow wave effects more and might not be pushed away from the strike trajectory of the snake and might therefore be captured more easily. Preliminary experimental support for this hypothesis was provided in a study by HAILEY & DAVIES (1986) who found a significant effect of prey size on the capture success of *Natrix maura*, with large fish indeed being captured more easily than small fish. Unfortunately, no quantitative models predicting how snake size or shape should affect the dynamics of prey capture have been proposed which would allow us to test these predictions.

Thus, we decided to empirically test the effect of snake and fish size on capture success (capture attempts and capture time) in *Natrix tesselata* (Laurenti 1768), a specialised aquatic snake that captures its prey under water using frontally directed strikes (LUISELLI & RUGIERO, 1991; FILIPPI et al., 1996; GRUSCHWITZ et al., 1999). Unlike the closely related *N. maura*, which includes both fish and frogs in its diet (GALAN, 1988; PLEGUEZELOS & MORENO, 1989; SANTOS & LLORENTE, 1998; SCHÄTTI, 1999; SANTOS et al., 2000), *N. tesselata* is a dietary specialist preying almost exclusively on fish (LUISELLI & RUGIERO, 1991; FILIPPI et al., 1996; GRUSCHWITZ et al., 1999).

MATERIALS AND METHODS

We used 9 *N. tesselata* (5 males, 4 females) in the experiments and classified 4 of them as being 'small' (mean±se: 34±2cm snout-vent length (SVL)), and 5 as being 'large' (mean±se: 51±2cm SVL). We measured SVL as the length from the tip of the snout to the posterior edge of the anal scute (POUGH & GROVES, 1983). Animals were housed in glass terraria (50x30x25cm) containing a sandy substrate, shelters, vegetation and a tub filled with water. Two 58-W fluorescent lamps suspended above the substrate provided heat and light for 9h per day. We fed the snakes goldfish, *Carassius auratus*, once weekly (Linnaeus 1758). Snakes were always eager to feed when placed in the experimental arena and did not show any signs of disturbance or stress.

TABLE 1

Prey size – snake size relationships for various snake species. All species shown have increasing average upper prey size limit with increasing snake size.

species	prey size-snake size relationship	diet	references
Alsophis cantherigerus	T	T	Henderson et al., 1988
Antillophis parvifrons	T	T	Henderson et al., 1988
Austrelaps labialis	T	T	Shine, 1987
Austrelaps ramsayi	T	T	Shine, 1987
Austrelaps superbus	T	T	Shine, 1987
Darlingtonia haetiana	T	T	Henderson et al., 1988
Drymobius chloroticus	T	T	Seib, 1984
Elaphe obsoleta	T	T	Weatherhead et al., 2003
Hypsirhynchus ferox	T	T	Henderson et al.,1988
Masticodryas melanolomus	T	T	Seib, 1984
Morelia spilota	T	T	Shine, 1991
Notechis scutatus	T	T	Shine, 1977
Pseudechis porphyriacus	T	T	Shine, 1991
Uromacer catesbyi	T	T	Henderson et al., 1988
Uromacer frenatus	T	T	Henderson et al., 1988
Uromacer oxyrhynchus	T	T	Henderson et al., 1988
Acrochordus arafurae	T	P	Shine, 1986
Regina grahamii	T	P	Godley et al., 1984
Regina septemvittata	T	P	Godley et al., 1984
Drymobius margaritiferus	S	T	Seib, 1984
Gloydius shedaoensis	S	T	Shine & Sun, 2003
Vipera latastei	S	T	Brito, 2004
Agkistrodon piscivorus	S	P	Vincent et al., 2004
Cerberus rynchops	S	P	Jayne et al., 1988
Enhydrina schistosa	S	P	Voris & Moffett, 1981
Laticauda colubrina	S	P	Shine et al., 2002
Laticauda frontalis	S	P	Shine et al., 2002
Natrix maura	S	P	Santos & Llorente, 1998
Nerodia fasciata	S	P	Miller & Mushinsky, 1990
Nerodia harteri	S	P	Greene et al., 1994
Nerodia rhombifer	S	P	Kofron, 1978; Plummer & Goy, 1984
Nerodia sipedon	S	P	King, 1993
Thamnophis atratus	S	P	Lind & Welsh, 1994

With regard to lower prey size limit, species that delete small prey from their diet when they grow are characterized by an ontogenetic shift (S); species that feed on large as well as small prey when getting larger, are categorized by an ontogenetic telescope (T). We distinguished piscivorous species (P) from species feeding on terrestrial prey (T).

In the experiments we used *C. auratus* as prey, ranging from 1 to 13g (i.e. 2 to 32%RPM (relative prey mass, i.e. prey mass divided by snake mass)) for both size classes of snakes. Although goldfish are not part of the natural diet of these snakes, other cypriniform fish are part of the natural diet of these snakes (Luiselli & Rugiero, 1991; Filippi et al., 1996; Gruschwitz et al., 1999). Fish were weighed with an electronic balance (Fx-3200, A&D, Johns Scientific Inc., Japan). Our prey range seemed sufficiently large, as the heaviest fish presented to the snakes were at times too large to ingest. We randomly retrieved fish from a large holding tank and presented each snake different sizes of fish spanning the entire range tested during the experiments. At the beginning of the experiments we placed a single *C. auratus* in a plexiglass aquarium (45x22x20cm) filled with water and a terrestrial section of 11cm wide. The water was kept at 25°C using a water heater. Subsequently, we introduced a snake into the aquarium and filmed it using a JVC digital video camera (Victor Company, Japan). We analysed the video-sequences to record the number of capture attempts and capture time for *N. tesselata* feeding on goldfish.

Capture attempts

We counted the number of strikes needed to capture a fish. When the snake released the fish after more than one second, the attempt was regarded as successful and subsequent capture attempts after releasing were not considered. Sometimes snakes performed multiple undirected strikes with jaws opened widely after an unsuccessful strike (see Bilcke et al., 2006). We did not include these strikes in the overall count.

Capture time

We defined capture time as the time elapsed between the first orientation of a snake towards a fish and the time when a snake grasped the fish with its jaws. Although this measure of capture performance includes a significant behavioural component on the part of both prey and predator, we do believe this is an ecologically relevant indicator of capture performance as both the approach to the prey and the actual strike determine the success and cost of a foraging attempt.

Statistics

We used Shapiro-Wilks tests to check the continuous data for normality before and after transformation. We $\log_{10}(x+1)$ transformed the number of capture attempts and capture time to obtain normal distributions (SOKAL & ROHLF, 1995). We identified five outliers using box plots and subsequently removed them from the dataset [i.e. extremely high values of capture attempts (49 and 32 attempts) and capture time (4669, 4033 and 3285sec)].

To test for the effect of snake and fish size on capture attempts and capture time, we used a mixed-model ANCOVA with snake size (small / large) as a fixed effect and fish mass as co-variable. We modelled individual (snake 1 to snake 9) nested within snake size as random effect to account for inter-individual variation in capture attempts and capture time. We performed the analyses using the proc MIXED procedure in SAS (LITTELL et al., 1996). Degrees of freedom of the fixed effects F-tests were adjusted for statistical dependence using Kenward Roger formulae. Residuals and estimates of the random effect of the final models were normal distributed (Shapiro-Wilks tests).

RESULTS

Since the relationship between fish mass, capture attempts and capture time did not differ between small and large snakes (i.e. no interaction effect: capture attempts: $F_{1,138}=2.63$, $p=0.11$; capture time: $F_{1,137}=0.78$, $p=0.38$), we omitted the interaction terms from the final models. We found significant inter-individual variation in the number of capture attempts and the capture time of the snakes (LR-test statistic, capture attempts: $\chi^2_1=49.8-45.7=4.1$, $p=0.04$; Fig.1a; capture time: $\chi^2_1=319.5-311.2=8.3$, $p=0.004$; Fig. 1b).

Large snakes needed more strikes to catch a fish (6 ± 1 and 5 ± 1 attempts, respectively) and spent more time capturing a fish than small snakes (685 ± 118s and 292 ± 42s, respectively). However, neither of these trends are significant (capture attempts: $F_{1,7.43}=0.85$, $p=0.38$; capture time: $F_{1,7.31}=1.88$, $p=0.21$). Snakes, however needed more strikes to catch larger fish ($F_{1,136}=3.77$, $p=0.05$; Fig. 1a). No relationship was found between fish size and capture time ($F_{1,135}=0.00$, $p=0.97$; Fig. 1b).

DISCUSSION

We tested the effect of snake and fish size on the capture success of the specialist aquatic snake *Natrix tesselata* to investigate hypotheses suggesting that 1) small snakes are better at catching fish than large snakes and 2) that larger fish are easier to catch than smaller fish. Unexpectedly, our study could not demonstrate significant effects of snake size on capture ability. Yet, despite significant inter-individual variation, we did find that small fish were significantly easier to capture than larger fish (in terms of the number of capture attempts needed, not in terms of capture time).

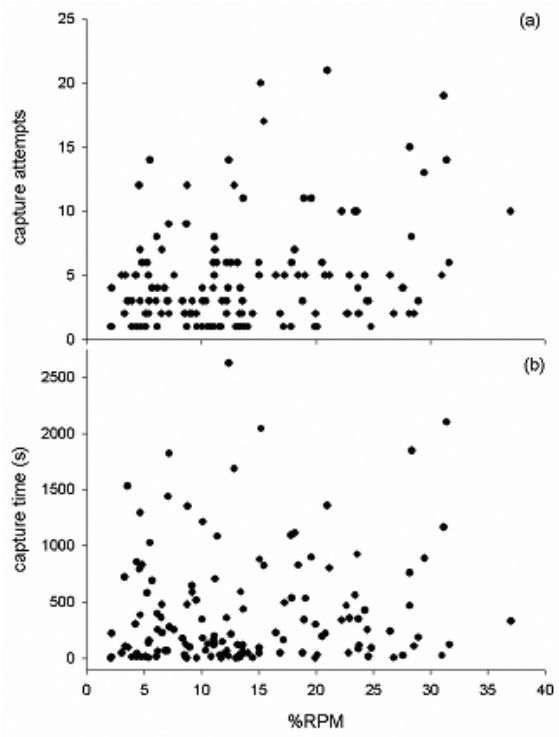

Fig. 1. – Relationship between the relative mass of goldfish (%RPM) with (a) the number of capture attempts and (b) capture time, for *Natrix tesselata*.

We found no significant effects of snake size on capture performance. Similar results were obtained by HAILEY & DAVIES (1986), suggesting that within the size range tested, snake size does not seem to have a big impact on capture performance. Maybe size effects become important only from a minimum size; i.e. there exists a minimum absolute size that will start to create disadvantages to prey capture in an aquatic environment. In support of this hypothesis, some generalist semi-aquatic snakes shift from aquatic prey to terrestrial prey (e.g. frogs) when they reach a specific size. For instance, *Nerodia erythrogaster* and *N. fasciata* shift from fish to frogs when they exceed 50cm SVL (MUSHINSKY et al., 1982). Also in *Rhabdophis tigrinus* and *Natrix maura* a shift from fish to frogs with increasing SVL was shown (MORIGUCHI & NAITO, 1982; SANTOS & LLORENTE, 1998). However, some species such as *Thamnophis validus* show the reverse trend: it shifts from frogs to fish when it exceeds 50-70cm SVL, suggesting that size effects might have complex interactions with prey capture behaviour or prey capture habitat (DE QUIEROZ et al., 2001). However, until we have a better understanding of the hydrodynamics and kinematics of aquatic prey capture in snakes it will be difficult to resolve these issues.

Our data also indicated no significant effects of fish size on capture time, but demonstrated large inter-individual variation. This observation may the consequence of our definition of capture time which includes an important behavioural component. For instance, capture time will depend on which foraging strategy is used: sit and wait or active foraging (BILCKE et al., 2006). Thus snakes

may use behavioural shifts when confronted with prey of different size to keep capture time constant. However, a significant effect of prey size was found on the number of capture attempts needed. Unexpectedly, and in contrast with data for *N. maura*, *N. tesselata* needs fewer attempts to catch small fish compared to large fish. Although these results appear contradictory at first, differences in the degree of aquatic specialization between the two snake species might lie at the basis for this observation. For example, a previous study showed that, despite similar foraging behaviours, *N. tesselata* has a higher capture success then *N. maura* (35% compared to 20%; BILCKE et al., 2006). Possibly, *N. tesselata* is morphologically better adapted to catch prey in the aquatic environment than *N. maura*. Indeed, compared to its more terrestrial congener *N. maura*, *N. tesselata* possesses a very narrow and streamlined head. Such a head shape is thought to reduce the hydrodynamic drag encountered during prey capture (YOUNG, 1991; HIBBITS & FITZGERALD, 2005). However, this needs to be assessed quantitatively. Why *N. tesselata* captures small fish more easily remains unclear at this point but may be in part due to differences in the escape response of large and small fish when confronted with a specialized aquatic predator such as *N. tesselata*. Clearly, further data on the kinematics and hydrodynamics of this prey-predator system are needed to better understand our results.

In conclusion, snake size does not affect the capture performance of *N. tesselata* within the size range tested. Possibly size becomes only important from a minimum absolute size, which is larger than the ones covered in our study. Moreover, *N. tesselata* needs fewer attempts to capture small fish, whereas *N. maura* needs less to capture big fish. Possible explanation lies in difference in morphology/kinematics between these species. An in-depth study of the hydrodynamics of this predator-prey system could provide ways to evaluate the importance of size effects.

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Elevational variation in body size of *Phrynocephalus vlangalii* in the North Qinghai-Xizang (Tibetan) Plateau

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ABSTRACT. We examined elevational and environmental aspects of body size variation in the Qinghai toad-headed lizard, *Phrynocephalus vlangalii*, using principal component analysis (PCA) of 9 morphological traits taken from 565 lizards from 17 populations. The first principal component (PC1) accounted for 67% of the size variation in males and 62% in females. For both males and females, PC1 decreased with increasing elevation. When analyzed in relation with respect to environmental variables, body size showed positive relationship with temperature, air pressure, and activity season length, but showed weaker or inconsistent relationships with rainfall and humidity. The described pattern is the converse of Bergmann's rule for this lizard species and suggests that this body size pattern is driven by temperature, air pressure or length of the activity season.

KEY WORDS : Altitudinal variation, Bergmann's rule, *Phrynocephalus vlangalii*, Body size

INTRODUCTION

Bergmann's rule predicts larger body size in colder areas and is assumed to be an adaptive response to environmental temperature (MAYR, 1956). Substantiated for endotherms (ASHTON et al., 2000; ASHTON, 2002; MEIRI & DAYAN, 2003), Bergmann's rule also holds for some ectothermic groups (ASHTON, 2002; ASHTON & FELDMAN, 2003), but this excludes squamates, which, in general, are smaller in colder areas (ASHTON & FELDMAN, 2003). Detailed studies of individual squamate species are necessary to better understand why they represent an exception to the general vertebrate pattern.

Here we evaluate body size changes in the Qinghai toad-headed lizard, *Phrynocephalus vlangalii,* across an elevational gradient in the Tibetan Plateau. Based on previous surveys (ASHTON & FELDMAN, 2003), we predict that *P. vlangalii* will decrease in body size with increased elevation and decreased temperature. Body sizes of lizards could also respond to other environmental factors, thus we also evaluate the effects of relative humidity, rainfall, oxygen pressure, and activity season length on body size variation. We predict shorter activity period and lower oxygen pressure could lessen net energy acquisition of lizards in high environments and have a negative impact on body size through reduced growth. Humidity and rainfall might also play a role in determining size variation in this arid-environment sand lizard.

Understanding the causal basis of geographic variation in body size has been the focus of much work in life-history evolution (STEARNS, 1992; ROFF, 2001). Elevational variation in body size is often correlated with environmental factors since body size is determined by both genetic and environmental factors (ENDLER, 1977). The relationship between variation in environmental gradients and the consequent variation in growth and body size has been of particular interest to evolutionary ecologists (ATKINSON, 1994; ATKINSON & SIBLY, 1997; ANGILLETTA & DUNHAM, 2003). Though these general patterns of body size variation relative to environmental factors have been well studied in endothermic vertebrates for many years (RENSCH, 1936; MAYER, 1963; NEVO, 1981; DUNHAM et al., 1989; BEAUPRE, 1995; ASHTON et al., 2000), relative to the large number that have focused latitude-based differences (ANGILLETTA et al., 2004; SEARS & ANGILLETTA, 2004). Ancestors of *Phrynocephalus* evolved into viviparous lizards during the uplifting of the Tibetan Plateau (WANG & MACEY, 1993; ZENG et al., 1997). With increasing elevation, air temperature becomes cooler, relative humidity increases, annual rainfall increases, and partial pressure of oxygen (pO_2) decreases (YOSHINO, 1975). Cooler environments could certainly promote evolutionary shifts (HEULIN et al., 1991; SHINE, 1995; ANDREWS, 2000; BLACKBURN, 2000; SURGET-GROBA et al., 2001). Because studies (LIAO et al., 2006) have indicated that morphological trait variation may have been influenced by the Tibetan Plateau uplift, *P. vlangalii* is a good model organism for studying body size evolution in response to variation of environmental factors across its broad elevational range from 2000 to 4600meters (ZHAO et al., 1999). We surveyed the size variation and explained the environmental factors that drive the pattern of body size.

MATERIALS AND METHODS

Specimens were collected from 17 populations in the North Tibet Plateau (Table 1) during the breeding period (from July to August of 2004). The following traits were measured: snout-vent length (SVL), total tail length (TL), head length (HL), head width (HW), head depth (HD), arm length (AL; distance between axilla and wrist), leg length (LL, distance between groin and ankle), distance between axillae (DBA) and distance between iliac crests (DBI). Specimens were preserved in the Lab of Zoology, School of Life Sciences, Lanzhou University. All traits

were measured to 0.1mm using vernier calipers. At sampled sites, elevation was measured by GPS.

Sex was determined based on the morphological descriptions of *P. vlangalii* (ZHAO et al., 1999). Female adult body size was based on the shortest SVL of a pregnant female. For males, the right testis, as well as part of the ductus epididymis, was dehydrated in ethanol, cleared in xylene, embedded in paraffin wax, sectioned at 8-10μm, and stained with Erlich's haematoxylin and eosin (HUMASON, 1967). Maturity in males was based on a testicular stage of 4-5 (VIEIRA et al., 2001) or presence of sperm.

Climatic data for the sampling areas of annual mean temperature, mean air pressure, mean rainfall and mean relative humidity was collected for all sampling areas from the Chinese National Climatic Data Center (CDC).

We used multivariate analysis of variance (MANOVA) to determine whether there were significant differences between the sexes. We performed principal component analyses (PCA) using the correlation matrix of the 9 morphological variables for each sex. Only PC1 was used into subsequent analyses, because it had an eigenvalue greater than 1.0. PC1 was essentially used as a surrogate for "size". We regressed the first principal component score (PC1) against elevation, air pressure, temperature, rainfall and humidity using least-square regression to determine whether clinal variation exists. All analyses were performed using population means and individual datapoints. All morphological data were expressed as mean ± S.E.M. Probability values below 0.05 were considered to be statistically significant.

TABLE 1

Morphological data: Mean ± S.E.M. of male (N=233) and female (N=332) adults in 17 populations of *Phrynocephalus vlangalii.* (SVL, snout-vent length; TL, tail length; AL, arm length; LL, leg length; HL, head length; HW, head width; HD, head depth; DBA, distance between axillae; DBI, distance between iliac crests; f, female; m, male).

Population	Elevation (m)	Sex	n	SVL (mm)	TL (mm)	AL (mm)	LL (mm)	HL (mm)	HW (mm)	HD (mm)	DBA (mm)	DBI (mm)
Guide	2289	f	10	60.8±1.5	63.3±0.8	9.4±0.2	26.0±0.4	15.5±0.3	13.8±0.2	10.2±0.2	12.5±0.3	8.8±0.2
		m	10	57.3±1.4	66.0±1.7	19.1±0.5	24.7±0.7	15.2±0.2	13.8±0.2	10.2±0.2	11.9±0.2	8.2±0.3
Tianzhu	2751	f	9	64.0±1.5	58.8±1.1	19.1±0.5	23.9±0.5	15.5±0.3	13.4±0.1	10.4±0.1	12.7±0.4	8.8±0.2
		m	7	63.3±1.1	65.0±1.2	20.5±0.6	25.3±0.5	15.9±0.2	14.2±0.2	11.0±0.3	12.3±0.3	8.8±0.2
Lenghu	2756	f	9	57.3±1.1	55.3±2.1	20.9±0.4	26.9±0.4	12.9±0.3	12.6±0.3	9.3±0.3	10.7±0.3	7.8±0.2
		m	6	55.6±2.0	53.0±2.7	20.6±0.7	26.3±0.9	12.9±0.3	12.6±0.4	9.1±0.3	11.1±0.5	7.7±0.5
Lumuhong	2857	f	6	59.0±3.3	65.8±3.7	21.5±0.8	28.2±0.6	14.0±0.8	14.5±0.7	9.8±0.4	11.2±0.7	6.5±0.4
		m	6	62.6±2.1	70.3±3.3	23.3±0.5	31.5±0.4	14.0±0.2	14.7±0.4	10.6±0.6	11.5±0.7	7.8±0.6
Delingha	2873	f	23	67.7±1.4	67.2±1.4	22.7±0.5	29.5±0.5	15.0±0.2	14.8±0.2	10.4±0.2	14.2±0.4	7.9±0.3
		m	17	67.3±1.0	73.0±1.2	23.2±0.6	31.3±0.5	15.2±0.2	15.2±0.2	11.2±0.3	14.6±0.2	8.1±0.3
Wutumeiren	2894	f	11	53.6±0.7	55.3±0.8	18.6±0.2	23.3±0.2	12.8±0.2	11.7±0.2	8.5±0.1	10.1±0.2	7.6±0.2
		m	9	56.2±1.5	62.1±1.6	19.1±0.5	26.2±0.3	13.9±0.2	12.5±0.2	9.4±0.2	10.2±0.1	7.5±0.1
Maqu	2926	f	32	57.8±0.6	56.6±0.5	15.9±0.2	21.9±0.2	14.2±0.2	13.2±0.1	10.1±0.1	11.6±0.2	8.1±0.1
		m	25	57.5±0.6	60.3±0.7	16.7±0.1	23.4±0.2	13.7±0.1	12.9±0.1	10.3±0.1	11.7±0.2	8.1±0.1
Wulan	2929	f	45	56.5±0.8	53.8±0.7	21.1±0.4	24.6±0.3	13.2±0.1	12.3±0.1	9.0±0.1	11.6±0.2	8.2±0.1
		m	18	56.6±1.4	61.1±1.6	22.0±0.6	26.6±0.7	13.4±0.4	12.7±0.4	9.8±0.3	11.8±0.3	7.7±0.2
Xiangride	3074	f	27	61.2±1.0	61.4±1.0	20.1±0.4	24.9±0.5	12.4±0.2	12.7±0.2	9.4±0.2	11.5±0.2	6.8±0.1
		m	9	59.0±1.7	63.9±1.7	19.1±1.1	24.0±0.8	12.1±0.4	12.6±0.4	9.5±0.4	10.5±0.4	5.7±0.3
Mangya	3174	f	8	56.4±1.5	55.6±1.1	21.3±0.5	25.5±0.5	13.5±0.3	12.7±0.3	9.9±0.4	11.5±0.2	8.3±0.2
		m	7	60.5±1.2	62.2±1.9	22.5±0.5	29.8±0.9	13.6±0.4	13.8±0.2	9.8±0.3	11.3±0.4	7.1±0.4
Doulan	3190	f	20	59.0±1.1	56.0±0.9	19.8±0.4	25.9±0.4	13.3±0.2	13.4±0.1	9.8±0.1	11.1±0.2	7.2±0.2
		m	17	54.1±1.0	56.8±1.4	19.3±0.3	26.0±0.5	13.3±0.2	12.6±0.2	10.0±0.1	11.2±0.2	7.3±0.2
Dachaidan	3200	f	8	55.3±1.9	52.5±3.4	19.1±1.0	24.9±1.2	12.8±0.4	12.3±0.2	8.8±0.2	10.6±0.4	7.3±0.1
		m	8	59.3±1.7	64.0±2.6	22.9±1.4	30.0±1.3	13.8±0.1	14.0±0.1	9.9±0.2	11.1±0.4	7.5±0.1
Ganzihe	3242	f	10	57.3±1.8	48.3±1.3	18.7±0.4	22.9±0.3	12.7±0.2	11.9±0.3	8.9±0.2	10.7±0.3	7.9±0.3
		m	14	57.3±1.3	54.5±2.0	19.2±0.4	24.9±0.5	13.4±0.2	12.7±0.2	9.8±0.2	11.2±0.2	7.8±0.1
Guinan	3370	f	19	54.6±1.1	54.2±1.4	18.7±0.4	25.3±0.6	13.4±0.2	12.2±0.2	9.0±0.2	11.5±0.3	7.6±0.2
		m	18	60.3±1.7	62.8±2.4	20.6±0.5	28.5±0.8	14.6±0.3	13.0±0.3	10.0±0.3	12.1±0.4	8.4±0.2
Xiaman	3470	f	14	61.2±0.8	59.8±0.6	20.6±1.2	27.3±1.5	14.4±0.2	13.9±0.3	10.5±0.1	12.2±0.3	8.2±0.1
		m	10	58.5±0.8	63.2±0.8	17.4±0.2	24.5±.0.3	14.4±0.1	13.3±0.2	10.3±0.2	11.8±0.2	7.9±.0.1
Maduo	4250	f	23	54.5±1.3	52.1±1.2	18.4±0.3	24.4±0.5	12.3±0.2	12.4±0.2	9.3±0.1	11.0±0.3	7.0±0.2
		m	19	54.1±0.8	56.6±0.8	19.0±0.3	25.0±0.5	12.9±0.2	12.6±0.2	9.8±0.1	10.5±0.2	6.0±0.2
Beiluhe	4565	f	58	52.7±0.5	49.4±0.6	17.8±0.2	23.6±0.2	12.0±0.1	11.7±0.1	8.5±0.1	10.7±0.1	7.1±0.1
		m	33	52.0±0.5	52.4±0.6	17.9±0.2	24.1±0.2	11.8±0.1	11.5±0.1	8.4±0.1	10.4±0.1	6.3±0.1

TABLE 2

Loading and the percentage of total variance explained for the first three principal components among 9 morphological traits of males (N=233) and females (N=332) of *Phrynocephalus vlangalii.*

Character	Male			Female		
	PC1	PC2	PC3	PC1	PC2	PC3
SVL	0.915	0.016	-0.019	0.877	-0.002	-0.161
TL	0.890	0.015	-0.174	0.836	0.057	-0.312
AL	0.721	0.583	0.149	0.697	0.528	0.323
LL	0.758	0.543	0.124	0.717	0.568	0.119
HL	0.868	-0.281	-0.084	0.833	-0.339	0.020
HW	0.880	-0.013	-0.301	0.860	-0.047	-0.262
HH	0.805	-0.269	-0.316	0.779	-0.235	-0.242
DBA	0.814	-0.010	0.263	0.836	-0.040	0.154
BDI	0.666	-0.109	0.525	0.606	-0.465	0.596
Total variance	66.747	11.109	6.783	61.916	11.059	8.318

RESULTS

The minimum adult male and female sizes were 49mm and 46mm. MANOVA with sex as the independent variable and the 9 morphological traits as the dependent variables indicated substantial sexual dimorphism in the *P. vlangalii* ($F_{9,555}$=21.21, P<0.001), so genders were separated for further analyses.

PC1 accounted for 66.7% of the variation in males, and all variables had positive loading values of 0.666 or above (Table 2). There was a significant linear regression (N=233, F=77.5, P<0.001 or N=17, F=6.9, P=0.019) with a decrease in PC1 score with increased elevation, with elevation accounting for 25.1% (N=233) or 31.7% (N=17) of the variation (Fig. 1A). PC1 accounted for 61.9% of the variation in females and all variables had positive loading values of 0.606 or above (Table 2). There was a significant (N=332, F=91.5, P<0.001; N=17, F=7.5, P<0.015) decrease in PC1 score with increased elevation, with elevation accounting for 21.7% (N=233) or 33.3% (N=17) of the variation (Fig. 1B). These results confirmed the hypothesis that both male and female lizards at higher elevations are smaller than at lower elevations.

TABLE 3

General linear regression was used depending on the relationship between the first principal component and each annual mean climatic factor. R^2, unstandardized coefficient, and constant of model, and significance of ANOVA analyses for testing the model were shown.

Factors	Sex	R^2	Slope	Constant	P
Air pressure	Male	**0.257**	0.001	-7.544	<0.001
(0.1BPa)	Female	**0.202**	0.001	-6.835	<0.001
Temperature	Male	**0.229**	0.018	-0.181	<0.001
(0.1°C)	Female	**0.238**	0.019	-0.207	<0.001
Rainfall	Male	0.059	-0.0001	0.316	0.004
(0.1mm)	Female	0.012	-0.0006	0.189	0.053
Humidity	Male	0.083	-0.026	1.309	<0.001
(%)	Female	0.027	-0.016	0.075	0.004

Significant positive linear regressions were found between PC1 scores and increased temperature, or air pressure, while significant negative regressions were found between PC1 scores and rainfall or relative humidity (Table 3). The exception to this was the lack of a significant regression between PC1 score and rainfall for females (P=0.053). On investigating the explanatory power of the models, we found that temperature (R^2: male, 0.229; female, 0.238) and air pressure (R^2: male, 0.257; female, 0.202) account for a larger proportion of the body size variation than rainfall (R^2: male, 0.059; female, 0.012) or relatively humidity (R^2: male, 0.083; female, 0.027).

DISCUSSION

Body size of *P. vlangalii* is positively correlated with temperature which is the converse of the pattern predicted by Bergmann's rule. Because some meteorological data are likely inter-correlated, making it difficult to discern between the different factors. However, it is not sufficient to evaluate Bergmann's rule through analysis of only one environmental factor. Consideration of the unique environment on the Tibetan Plateau suggests at least three possible effects on body size: temperature, hypoxia and food shortage caused by shorter activity time at higher elevations.

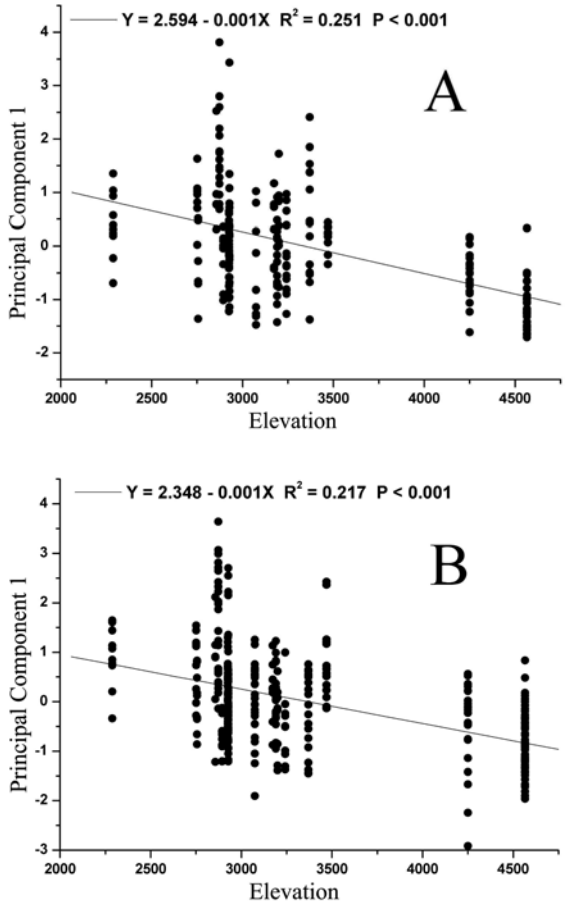

Fig. 1. – Regression analysis plots of the first principal component (PC1) on elevation for (A) males and (B) females.

The validity of Bergmann's rule in ectotherms has been widely questioned (Mousseau, 1997; Blackburn et al., 1999; Belk & Houston, 2002). In particular it has been noticed that lizards in colder environments may grow to a smaller size (Ashton & Feldman, 2003). The traditional heat conservation explanation proposed (Bergmann, 1847; Mayer, 1963) does not apply to these ectotherms (Ashton & Feldman, 2003). Temperature affects the physiological and behavioural performance of ectothermic vertebrates (Navas, 2003). Thermal constraints on ectotherm activity is directly related to the available environmental temperature (De Witt, 1967; Grant & Dunham, 1990). Even when distributed across a wide range of thermal environments, lizards might exhibit only a small and surprisingly consistent range of body temperatures (Bogert, 1949; Andrews, 1998). Consistent patterns of temperature variation are not necessarily associated with elevation within species due to behavioural compensation (Sears & Angilletta, 2004). Behavioural thermoregulation could conceivably be an important buffering mechanism in this small lizard, e.g., shuttling among thermally inhomogeneous patches, though this is thought to be more important in large ectotherms with larger thermal inertia (C. R. Peterson, personal communication, Grant, 1990). Higher body temperatures could help with digestion and development in cooler and unstable environments because most squamates swallow food items whole and retain young for long periods (Ashton & Feldman, 2003). Increased selected body temperature (SBT) by lizards could increase metabolisable energy intake during digestion (Brown & Griffin, 2005), but without optimal warm environments, growth rates of lizards (Dunham et al., 1989) are limited by the rates at which food items passed through the gut. However, energy consumption will be increased by higher body temperatures in an active iguanid lizard relative to an inactive one (Dawson, 1975; Grant, 1990). Therefore there is clearly a trade-off between benefits from thermoregulation activity, such as optimal food assimilation in cooler environments and energy consumption of activity.

At an altitude of 4000m (13,200ft) the concentration of oxygen in 1 liter of inspired air is 21% oxygen, just as at sea level, but because of the lower barometric pressure, 1 liter of air at 4000m contains just 63% of the number of oxygen molecules at sea level (Beall, 2000), which leads to hypoxia in animals. Hypoxia is the most prominent stress that populations living at high elevations must deal with (Hammond et al., 2001). Animals at higher elevations must adapt to the stress of limited oxygen availability relative to lower elevation and still sustain aerobic metabolic processes. For example, the oxygen consumption of animals will show a drop under hypoxic conditions (van den Thillart et al., 1992) and this reduces the amount of oxygen available to the tissue (Moran, 1982). Here, the same quantity of food consumed will produce less energy than in normal conditions. However, animals living at high elevations generally have increased energy demands and energy intake and so may experience limitations to aerobic activities such as exercise and heat production due to the lower oxygen availability (Snyder, 1981; Chappell et al., 1988). This is not conducive to increased growth. Organisms may have metabolic rates below normal resting level in response to stressful environmental conditions (Gillooly et al., 2001). This provides a problem in that metabolic rates of reptiles decrease under low oxygen pressure (Thompson et al., 1995; Zari, 1996; Sears, 2005) and low temperature (Kam, 1993; Storey, 1996; Hicks & Wang, 2004), but the rate of energy expenditure per unit mass increases with decreased body size (Peters, 1983). Lizards could therefore benefit from the increased metabolic rate per unit mass to help increase body temperature in cooler environments. *P. vlangalii* has to balance the conflict of hypoxia and lower temperature by maintaining a relatively constant body temperature during activity. Smaller body size appears to be one adaptation that contributes to this.

Daily and seasonal activity periods for a given ectotherm at lower elevations are longer than those for the same ectotherm at higher elevations (Masaki, 1967; Grant & Dunham, 1990). It seems that higher elevational environments could lessen seasonal activity periods, and potentially lessen the available annual forging times and consequently the net energy uptake for an animal at high elevations. This could also lead to a decrease in body size. This pattern has been reported in invertebrates (Mousseau & Roff, 1989) and has been predicted for lizards (Adolph & Porter, 1996; Montgomery et al., 2003).

In conclusion, this study showed that the body size of *P. vlangalii* decreased with increasing elevation. Because temperature decreases with elevation, this represents the converse of Bergmann's rule, and shows that this is not universally valid for interpreting animal body size clines. We attribute this decline to temperature, hypoxia and food shortage caused by shorter activity periods. Different patterns of energy consumption and energy distribution appear to have different effects on body growth, at different elevations.

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Calling activity of *Crossodactylus gaudichaudii* (Anura: Hylodidae) in an Atlantic Rainforest area at Ilha Grande, Rio de Janeiro, Brasil

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ABSTRACT. The calling activity in anuran amphibians can be influenced by several environmental variables that can affect such activity in different ways. In the present study we investigated the relationship of the daily calling activity of males of the diurnal frog *Crossodactylus gaudichaudi* with some environmental variables. The study was carried out between July 2003 and June 2005 in an area of Atlantic forest in Ilha Grande, an island located on the southern coast of the State of Rio de Janeiro. The observations were made in three fixed points in the forest, adjacent to streams, always from 05:00 to 19:00h, one day per month. We verified that the males of *C. gaudichaudii* have strictly diurnal calling activity, staying active during all months of the year. The highest counts of emitted calls were registered, in general, at the beginning and at the end of each day sampled. Air temperature, relative humidity and light intensity affected the daily rate of calling activity in different ways, with air temperature and light intensity seeming to be the factors that influence most importantly the activity of the species. The photoperiod seemed to be the main factor regulating the extension of the calling activity along the year in males of *C. gaudichaudii*.

KEY WORDS : Calling activity, Atlantic Rainforest, streams, *Crossodactylus gaudichaudii*.

INTRODUCTION

Calling behaviour in anurans constitutes the main form of communication for these organisms, followed by visual signals (WELLS, 1977; DUELLMAN & TRUEB, 1986). In most anuran species the calls are essential to guarantee the reproductive success, being used to attract females (mainly), to defend a territory, and to display distress (e.g. DUELLMAN & TRUEB, 1986; WELLS, 1988; HADDAD & GIARETTA, 1999; GUIMARÃES & BASTOS, 2003). However, calling activity involves energetic costs and increased exposure to predation by calling males (DUELLMAN & TRUEB, 1986; JUDGE & BROOKS, 2001; WONG et al., 2004).

Calling activity of frogs is influenced by environmental factors such as photoperiod (e.g. JAEGER et al., 1976; WHITTIER & CREWS, 1987), light intensity (e.g. HATANO et al., 2002), relative humidity (e.g. CREE, 1989; HATANO et al., 2002) and air temperature (e.g. LICHT, 1969; NAVAS, 1996). However, the relative importance of these factors on calling activity of males may differ among species (BROOKE et al., 2000). For the Atlantic Rainforest biome of eastern Brazil, there are few studies relating the calling activity to local environmental factors (BOQUIMPANI-FREITAS et al., 2002; HATANO et al., 2002; VAN SLUYS et al., 2006).

Frogs of the genus *Crossodactylus* are diurnal and live in rocky streams inside the forest (WEYGOLDT & CARVALHO-E-SILVA, 1992; IZECKSOHN & CARVALHO-E-SILVA, 2001; JORDÃO-NOGUEIRA et al., 2006). C*rossodactylus gaudichaudii* Duméril & Bibron (1841) is commonly found associated with streams inside the forest in the states of Rio de Janeiro and São Paulo (IZECKSOHN & CARVALHO-E-SILVA, 2001). During the day, males of *C. gaudichaudii* communicate by acoustic and visual signals (WEYGOLDT & CARVALHO-E-SILVA, 1992). However, the lack of studies specifically addressing aspects of the biology of *C. gaudichaudii* limits our understanding of parameters of the species ecology and natural history. In the present study we investigate the calling activity of *C. gaudichaudii* in the Atlantic Rainforest of Ilha Grande and try to evaluate how some local environmental factors affect the calling activity of males.

MATERIALS AND METHODS

Study area

Data was gathered from July 2003 to June 2005 in the Atlantic rainforest of Ilha Grande (23°11' S, 44°12' W), a large continental island (aproximately 19000ha) on the southern coast of Rio de Janeiro state, southeastern Brazil. The forest exhibits different levels of disturbance caused by human activities during the last century (ARAÚJO & OLIVEIRA, 1988). Annual rainfall at Ilha Grande is about 2200mm, and mean annual temperature is about 23°C (HATANO et al., 2002). The study site is located on a trail between Vila Dois Rios and the Caxadaço beach, at the seaward side of the island.

Collecting methods and analysis

We established three observation sites to record frog activity at three small forest streams located > 100m apart. The observations were made always from 05:00 to 19:00h, one day per month, between July 2003 and June 2005. We considered only the individuals that were calling within a perimeter of approximately five meters around the three sampling sites.

At hourly intervals, we registered for five minutes, at each point, the number of individuals calling and the number of calls emitted (with a hand counter and a chro-

nometer). At the beginning of each hour we measured the light intensity (in lux, with a luxmeter), and the air temperature (°C) and the relative humidity (%) (with a thermohigrometer). Besides, we obtained the photoperiod (in minutes) for the respective days of sampling (HATANO et al., 2002). Frog calling activity is expressed as the mean number of calls at the sites and as the mean number of active individuals per hourly and monthly intervals.

To estimate the extension (in minutes) of the calling activity, we recorded the time of the beginning and the end of frog activity in each day sampled. The onset and end of frog activity were considered as the time when the first and last calls were recorded, respectively.

The mean number of calls and the mean number of active individuals per hour was regressed against air temperature, air humidity and light intensity recorded at the same period using multiple regression analysis (fixed model). In this case, we analyzed data for the dry (April to September) and rainy (October to March) seasons separately. To test for differences between the mean number of calls emitted during the months of the dry and rainy seasons we used a one-way ANOVA (ZAR, 1999).

The photoperiod was regressed against the extension of the calling activity using regression analysis (ZAR, 1999). To evaluate the effect of light on the onset and end of *C. gaudichaudii* calling activity, we related the precise time of the start and the end of frog activity to the hour of sunrise and sunset of the same day, respectively, using regression analysis (ZAR, 1999). In order to evaluate if any nocturnal activity occurred, observations were made for 24h during two months of a dry season (July and August 2003) and two months of a rainy season (December 2003 and March 2004).

RESULTS

We did not record nocturnal activity by males of *Crossodactylus gaudichaudii*. The males called during all months of the study period at Ilha Grande. The daily calling activity was most intense, generally, at the beginning (from 05:00-06:00h) and at the end (from 17:00-18:00h) of the light period (Figs 1 and 2). The daily calling activity usually started between 05:00 and 06:30h and ended between 17:00 and 18:30h, depending on the period of the year (Figs 3 and 4). At Ilha Grande, the photoperiod differs about 2.5h between the longest and the shortest days, a value similar to that observed for the difference between the smallest and the highest values of calling activity extension in *C. gaudichaudii* (Fig. 5).

In the dry season, a multiple regression analysis showed an overall significant effect of environmental parameters on the mean number of calls during the day (R^2=0.101; $F_{3,164}$=4.813; P=0.003). However, only air temperature (t=1.969; P=0.05) and light intensity (t=3.394; P=0.001) explained an additional part of the mean number of calls after removing the effect of the other variables. The multiple regression analysis did not show an overall significant relation to mean number of calling individuals (R^2=0.1; $F_{3,164}$=0.512; P=0.674). In the rainy season, the environmental factors showed a significant effect on mean number of calls (R^2=0.070; $F_{3,164}$=3.651; P=0.014), but only air temperature (t=2.544; P=0.005) and relative humidity (t=2.943; P=0.003) explained an additional part of the relationship after removing the effect of the other variables. Similarly, the environmental factors interacted to affect the mean number of calling individuals (R^2=0.071; $F_{3,164}$=3.721; P=0.013), with air temperature (t=2.997; P=0.003) and relative humidity (t=3.108; P=0.002) showing an additive effect. The mean number of calls and the mean number of calling individuals on each month did not differ between the rainy and wet seasons (ANOVA: $F_{1,22}$=1.011; P=0.326 and $F_{1,22}$=1.021; P=0.322, respectively).

The start and the end of *Crossodactylus gaudichaudii's* activity in each month were usually coincident with the sunrise and sunset, respectively. The start of calling activity was positively related to the time of sunrise (R^2=0.645; $F_{1,22}$=40.013; P<0.001). Similarly, the end of frog activity was positively related to the time of sunset (R^2=0.703; $F_{1,22}$=52.145; P<0.001). The relationship between day length and extent of frog activity was positive and significant (R^2=0.879; $F_{1,22}$=159.211; P<0.001).

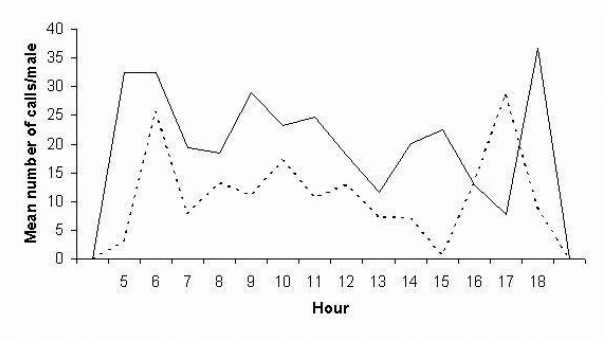

Fig. 1. – Mean number of calls emitted by males of *Crossodactylus gaudichaudii* in the three sampled points in the forest between 05:00 and 19:00h in months of dry (broken line) and rainy (continuous line) seasons in a Atlantic Rainforest area at Ilha Grande.

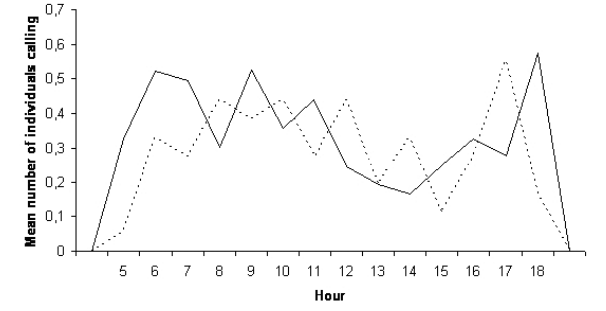

Fig. 2. – Mean number of calling individuals of *Crossodactylus gaudichaudii* in the three sampled points in the forest between 05:00 and 19:00h in months of dry (broken line) and rainy (continuous line) seasons in a Atlantic Rainforest area at Ilha Grande.

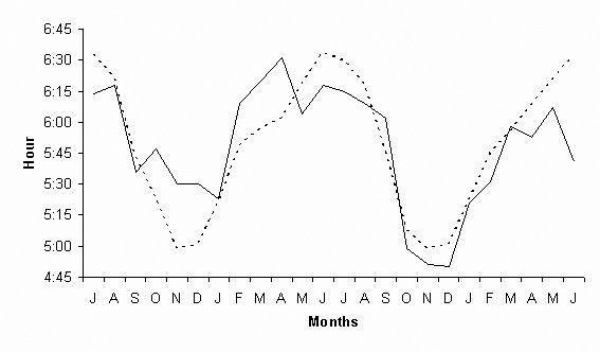

Fig. 3. – Time of beginning (continuous line) of *Crossodactylus gaudichaudii* calling activity and time of sunrise (broken line) between July 2003 and June 2005 in a Atlantic Rainforest area at Ilha Grande.

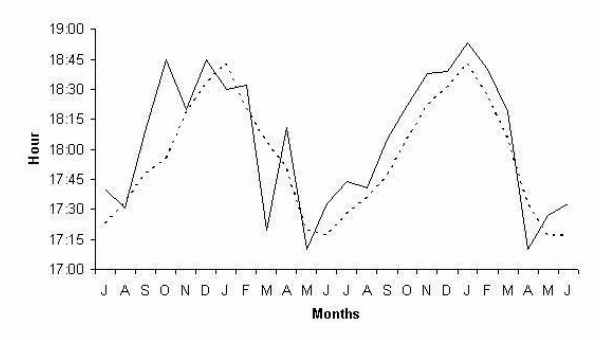

Fig. 4. – Time of end (continuous line) of *Crossodactylus gaudichaudii* calling activity and time of sunset (broken line) between July 2003 and June 2005 in a Atlantic Rainforest area at Ilha Grande.

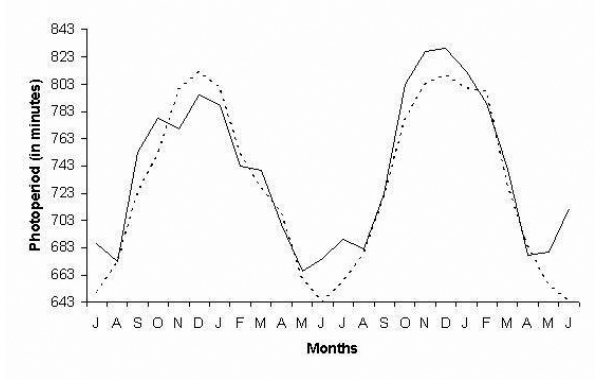

Fig. 5. – Calling activity extension, in minutes, of *Crossodactylus gaudichaudii* (continuous line) and daylength (broken line) between July 2003 and June 2005 in a Atlantic Rainforest area at Ilha Grande.

DISCUSSION

The data of the present study indicate a strictly diurnal calling activity for *Crossodactylus gaudichaudii*. This corroborates the previous available informations for the species, which are based on sporadic observations (WEYGOLDT & CARVALHO-E-SILVA, 1992; IZECKSOHN & CARVALHO-E-SILVA, 2001). Although most anuran species are nocturnal, males of *C. gaudichaudii* and of other species that live in habitats hypersaturated by humidity (having water available during throughout the year) would likely be less subjected to dehydration than other anurans. This may possibly have allowed the evolution of a diurnal activity pattern (HADDAD & GIARETTA, 1999; BOQUIMPANI-FREITAS et al., 2002).

At the Atlantic Rainforest of Ilha Grande, air temperature significantly affects the calling activity of *C. gaudichaudii*. This variable represents an important factor affecting the calling activity of anurans in general, and in some cases this is the most important factor regulating their activity (POUGH et al., 1983; NAVAS, 1996). For the hylodid *H. phyllodes*, HATANO et al. (2002) found that air temperature also significantly affected the calling activity of males.

Relative air humidity can limit the activity of anuran amphibians both temporally and spatially (e.g. CREE, 1989). On the other hand, air humidity helps in transmission of the sound of the calls, as the resonant transmission is more efficient in humid air than in dry air (HARRIS, 1966). However, although air moisture affects activity of several anurans species, its effect should be less pronounced in species living permanently associated with streams such as *C. gaudichaudii* (WEYGOLDT & CARVALHO-E-SILVA, 1992; IZECKSOHN & CARVALHO-E-SILVA, 2001). In this case, even in the drier months, the atmosphere's own humidity would likely be enough to allow calling activity of the frogs without risks of desiccation.

The relative humidity also affected significantly the calling activity of *Hylodes phyllodes* (HATANO et al., 2002), which lives syntopically with *C. gaudichaudii* in some streams in the Atlantic forest of the Ilha Grande. Conversely, for the sympatric cyclorhamphid *Proceratophrys appendiculata* (BOQUIMPANI-FREITAS et al., 2002), the relative humidity of the air did not affect the calling activity in a significant way. This suggests that even for species living in streams air humidity has different effects.

The light intensity is as an important environmental variable which influences the calling activity of anuran amphibians (JAEGER et al., 1976; PANCHARATNA & PATIL, 1997). For another strictly diurnal hylodid species of the Atlantic forest, the light intensity also constitutes an important factor affecting the calling activity (HATANO et al., 2002). In our study, light intensity also constitutes an important environmental variable affecting the calling of males of *C. gaudichaudii*. This indicates the importance of that environmental factor for the calling activity in diurnal frogs species.

The hourly calling activity of *C. gaudichaudii* at Ilha Grande was bimodal. The emission of calls by males involves energy expenses and exposes them to greater risks of predation (DUELLMAN & TRUEB, 1986; JUDGE & BROOKS, 2001; WONG et al., 2004). The calling activity was more intense, in general, at the beginning and at the end of the day, periods in which the light intensity was comparatively reduced in our study. An increase in calling activity during the hours with smaller light intensity can be advantageous, because it can reduce the chances of

desiccation of the skin and of a calling male to be detected by visually oriented predators (OSEEN & WASSERSUG, 2002).

Because in our study light intensity significantly affected the calling activity in the dry season, we suggest that this variable (which had a clear effect in both seasons) and air temperature constitute the main factors affecting the calling activity of *Crossodactylus gaudichaudii* along the day. Those two variables have been suggested as important factors limiting or even suppressing the activity of anuran amphibians (CARDOSO & HADDAD, 1992).

Our data show that the males of *Crossodactylus gaudichaudii* in the Atlantic forest of the Ilha Grande emitted calls during all months of the year, indicating that the species has an extensive or even continuous reproductive activity, potentially allowing the occurrence of different reproductive events along the same year. Similar results were found for species of the genus *Hylodes* (e.g. HADDAD & GIARETTA, 1999), which is closely related to the genus *Crossodactylus*. Additionally, the absence of differences in calling activity between the dry and rainy seasons reinforces the suggestion that there is no seasonality in the reproductive activity of the species.

The coincidence among the times of sunrise and sunset with the start and the end of calling activity of males of *Crossodactylus gaudichaudii* is a clear indication of the strictly diurnal activity of this species. Day length also regulates the extent of calling activity of this frog. This has been observed for other hylodid species (HATANO et al., 2002), for which the extent of activity is regulated by the extent of the light period. The photoperiod is an important factor affecting the activity of several species of amphibians (JAEGER et al., 1976; PANCHARATNA & PATIL, 1997). In our study, the photoperiod explained a considerable portion (approximately 88%) of the extent of the calling activity. The difference observed between the shortest and longest days was similar to that observed for the shortest and longest durations of calling activity, suggesting a narrow relationship between these two factors. As well as in other studies with diurnal species (HATANO et al., 2002), the extent of the calling activity thus seems to be regulated by the length of the day.

We conclude that males of *Crossodactylus gaudichaudii* have a strictly diurnal activity and are active during all months of the year. Air temperature, relative humidity and light intensity seems to interact in the dry and rainy seasons to affect the daily activity of this species, with air temperature and light intensity seeming to constitute the most important factors influencing calling frog activity. Photoperiod is the main factor regulating the extent of the calling activity along the year.

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The use of demecolcine for enucleation of bovine oocytes

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ABSTRACT. Nuclear transfer requires removing all genetic materials associated with the chromosomes of recipient oocyte. This Study was designed to further explore the pharmacological role of Demecolcine (DM) on assisting enucleation in animal somatic cell nuclear transfer. The *in vitro* matured bovine oocytes were incubated with DM at different concentration, or at a fixed DM concentration for additional different hours, then the oocytes extrusion cones rate (ECR) were determined in the inverted microscope. The highest ECR (61.90%) was measured from the treatment with 0.5μg/mL demecolcine. The time-dependent manner of the development of the extrusion cones, in 2hr groups were significantly higher ($P<0.05$) than in 0hr, 0.5hr, 1hr and 2.5hr groups. The highest ECR were found *in vitro* matured 18hr groups (73.86%), which was significantly higher ($P<0.05$) than that observed in 14hr, 16hr groups, and 20hr groups. However, the Granualar cell existence during maturation can influence on PR and embryos development rate. The *in vitro* matured 18hr bovine oocytes with granualar cell were added to DM, which were significantly higher ($P<0.05$) than that of control groups. Meanwhile, we evaluated the effects of DM on the cleavage rate of *in vitro* matured oocytes. The results showed that the IVM medium with or without DM, the IVF embryos rate of cleavage, of blastocysts, and average cell number of blastocysts between the two groups were not significantly different from each other. This simple, chemically assisted method to remove maternal chromosomes makes it possible to produce a large number of nuclear-transferred eggs and to efficiently produce cloned bovines.

KEY WORDS : bovine, assisted-enucleation, Demecolcine, nuclear transfer, oocyte

INTRODUCTION

Since the birth of Dolly in 1997 (WILMUT et al., 1997), a number of studies have reported the production of cloned animals (WILMUt et al., 1997; CAMPBELL, 2002; LI et al., 2004; TIAN et al., 2003; WILMUT & PATERSON, 2003). These successes revealed the extraordinary capacity of the oocyte to erase the process of genome cell differentiation and to reprogram the genetic information to produce a new individual. This process is attainable by nuclear transfer, which consists of the replacement of the maternal genetic material of the oocyte by the genetic information of donor cells after nuclear transfer. Unfortunately, even if cloning is possible in these few experiments, nuclear transfer is a very complex, time consuming, poorly understood and inefficient process. In fact, the efficiency of nuclear transfer has been estimated to be between 1 and 2% of all oocytes used (POLEJAEVA et al., 2000). The reasons for this low efficiency can be attributed to the source and quality of oocytes, the preparation of the recipient cytoplasm, the donor cell type, the synchronization of the cell cycle of both, recipient cytoplasm and donor cells, the failure to reprogram the transplanted nucleus and finally the failure of artificial activation methods. Clearly, much research on cloning remains to be done.

One of the major steps involved in the nuclear transfer procedure is the removal of genetic material from the recipient oocyte (enucleation). It is clear that this procedure also removes important cytoplasmic components, which may reduce cytoplast viability. In routine nuclear transfer procedures, one third or more of the ooplasm is frequently removed that would presumably result in a corresponding decrease in the total cell number of cloned blastocysts. This procedure requires time and limits the number of oocytes available for cloning. In present study, our aim was to evaluate the utility of demecolcine to assisted-enucleation of bovine oocytes. In addition, the developmental competence of resulting cytoplasts was examined in nuclear transfer experiments using fibroblast as nuclear donors. We examined the developmental competence of DM treatment using IVF.

MATERIALS AND METHODS

1. Chemicals and materials

Dishes for oocyte culture were purchased from Corning/Coster Company (NY, USA), and all chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2. Cell Cultures

Primary cultures of bovine ear skin fibroblasts were established from tissue samples of a 3-week-old Holstein cow. Briefly, the tissues were cut into small pieces and dispersed by exposure to 0.1% (w/v) trypsin (Gibco, Grand Island, NY). The cell suspension was then transferred into 10cm culture dishes containing Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (Hyclone FBS; Biochrom, Berlin, Germany), 2mM L-glutamine, 0.1mM 2-mercaptoethanol, 2mM non-essential amino acids (Sigma, St.

Louis, MO), 100IU/mL penicillin and 100μg/mL streptomycin (passage 0). The cells were cultured until subconfluence (usually 2–3 days) at 37°C in a humidified atmosphere of 5% CO_2 in air and then frozen in DMEM with 10% DMSO and 20% FBS.

3. Preparation of Donor Cells

Frozen-thawed adult ear fibroblast cells less than four passages were used as donor cells, which were plated into a four-well culture dish, and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin until confluent. These cells were then subjected to serum starvation (0.5% FBS in DMEM) for 5–8 days as described by (WILMUT et al., 1997). Immediately prior to somatic cell nuclear transfer (SCNT), donor cells were collected after trypsinization and then resuspended in DMEM containing 0.5% FBS.

4. Oocyte Collection and Removal of Cumulus Cells

Bovine ovaries were collected immediately after slaughter and brought to the laboratory in normal saline at 25-32°C within 4hr after removal. Cumulus-oocyte complexes (COCs) aspirated from small antral follicles were washed three times with Phosphate buffer fluid supplemented with 5% FBS, 0.2mM sodium pyruvate (Sigma) and 50μg/mL gentamycin sulfate (Sigma). The oocytes were then cultured for 18 to 20hr in HEPES buffered TCM 199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS, 0.02units/mL FSH (from porcine pituitary, Sigma), 1μg/mL estradiol 17 ß 0.2mM sodium pyruvate, and 50μg/mL gentamycin sulfate under a humidified atmosphere of 5% CO_2 in air at 38.5°C. Cumulus cells were removed by vortexing the COCs in 0.2% hyaluronidase (Type 1-S, Sigma) in Ca^{2+}- and Mg^{2+}- free Dulbecco's PBS. To visualize DNA, oocytes were stained in 5μg/mL Hoechst 33342 for 10min and observed with a Nikon inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan) under normal and/or ultraviolet (UV) light.

5. Enucleation, Somatic Cell Microinjection

In the groups treated with DM media, bovine oocytes were enucleated according to a modified protocol. Briefly, as shown in Fig.1, eggs with extrusion cones membrane were moved to medium supplemented with 5mg/mL cytochalasin B (CB) and 0.5mg/mL demecolcine and then oocytes were fixed to the holding pipette and the extrusion cones membrane was removed by aspiration using a pipette with a diameter of 22μm. After enucleation, the donor cell was introduced through the same pipette in the zona pellucida and wedged between the zona pellucida and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. After injection, reconstructed embryos were cultured in SOFaa medium until fusion.

6. Fusion, Activation, and Embryo Culture

Fusion was done by a double electric pulse of 2.1kV/cm for 10msec using a Zimmermann Cell Fusion Instrument (Bachofer, Reutlingen, Germany). Reconstructed embryos were cultured in SOFaa (NaCl 107.63mM, KCl 7.16mM, KH_2P0_4 1.19mM, $MgSO_4$ 1.51mM, $CaCl_2.2H_20$ 1.78mM, Sodium lactate 5.35mM, $NaHCO_3$ 25.00mM, Na-pyruvtae 7.27mM, L-Glutamine 0.20mM, BME amino acdis 45.0μL/mL, MEM amino acids 5.0μL/mL, tri-Sodium-citrate 0.34mM, Myo-inositol 2.77mM, Gentamycine 50.0μg/mL, Phenol-red 10μg/mL) medium supplemented with 2mg/mL BSA. Only fused embryos were used for the activation experiments to avoid incorrect interpretation of the PB extrusion.

The reconstructed embryos were activated with 5μM ionomycin in PBS for 5min, followed by exposure to 30mg/mL BSA for 4min in order to stop the activation. To maintain the low level of MPF, the eggs were further incubated in IVC medium containing 2μM DMAP for 4hr. The reconstructed embryos were cultured in 0.5mL SOFaa, supplemented with 5% FBS, in 4-well dishes overlaid with paraffin oil at 38.5°C in a humidified atmosphere with 5% CO_2. Morphological survival and cleavage rates were recorded. All cleaved embryos were further cultured in SOFaa medium supplemented with 2mg/mL BSA for 7d.

7. *In Vitro* Fertilization

After maturation for 20hr, oocytes with expanded cumulus complexes were removed from maturation medium, washed three times in TL Hepes (Bio-Whittaker, Walkersville, MD, USA), aliquoted into groups of 15-20 and washed three times in fertilization medium, and then transferred into a 50μL drop of fertilization medium in a petri dish (Becton Dickinson, Franklin Lakes, NJ, USA) and placed under mineral oil in 5% CO_2 in humidified air at 38.5°C. Semen from the same bull was used in this study, and was thawed at 38°C for 1min. The sperm was washed three times by centrifugation at 453×g for 8min in sperm wash medium. Following the final wash, the sperm motility and concentration were determined. Sperm pellets were re-suspended in sperm wash medium to a volume of 1mL. Sperm suspension was added to each fertilization drop, giving a total concentration of 1.0×10^6 spermatozoa/mL. Oocytes and sperm were incubated together in 5% CO_2 in humidified air at 39°C for 6hr before *in vitro* culture.

8. Experimental Design

Experiment 1: This experiment was designed to define the optimal concentration of DM for initiating extrusion formation of bovine oocytes. Oocytes at the MII stage were cultured in SOFaa plus 10% FBS containing 0; 0.4; 0.5 or 0.6μg/mL at 38.5°C for 2hr. Projections were recorded and enucleated under an inverted microscope.

Experiment 2: This experiment was designed to investigate whether exposure time has any effect on the formation of extrusion cones. Oocytes were incubated 0; 0.5; 1; 1.5; 2 or 2.5hr, respectively.

Experiment 3: This experiment was designed to examine the effects of oocyte maturation time on the formation of extrusion cones. Oocytes were matured 14hr, 16hr, 18hr and 20hr, respectively and then incubated 2hr with 0.5μg/mL DM .The treatment was similar to experiment 1.

Experiment 4: This experiment was designed to check if granualar cell existence during maturation can influence oocytes maturation if DM existed in the media. DM was added into the media when oocytes were cultured at 14, 16 or 18hr, and oocytes of all groups were cultured for 20hr.

Experiment 5: This experiment was designed to check the effect of oocyte developmental capacity in the presence or absence of DM. DM was added to oocytes maturated for 18hr and IVF at 20hr.

9. Statistical Analysis

The formation, cleavage and blastocysts rates were compared by χ^2 analysis. Differences at $P<0.05$ were considered significant.

RESULTS

After DM treatment, the oocytes had a membrane extrusion cone in which the chromosomes were located, and the extrusion cone was close to Pb 1 (Fig. 1A, B). The maternal chromosomes were easily aspirated and only about 5% volume of cytoplasm was removed. The metaphase II oocytes were treated with different concentrations of demecolcine (ranging from 0.4 to 0.6μg/mL) for 2hr, the extrusion cone was affected in a dose-dependent manner as shown in Table I. On examination under UV light, the extrusion cone gathered with chromosomes in all of the oocytes (100%) (Fig.1A, B). Recipient cytoplasts for NT experiments were prepared by demecolcine treatment. NT embryos were produced by fusion with fibroblast cells less than four passages.

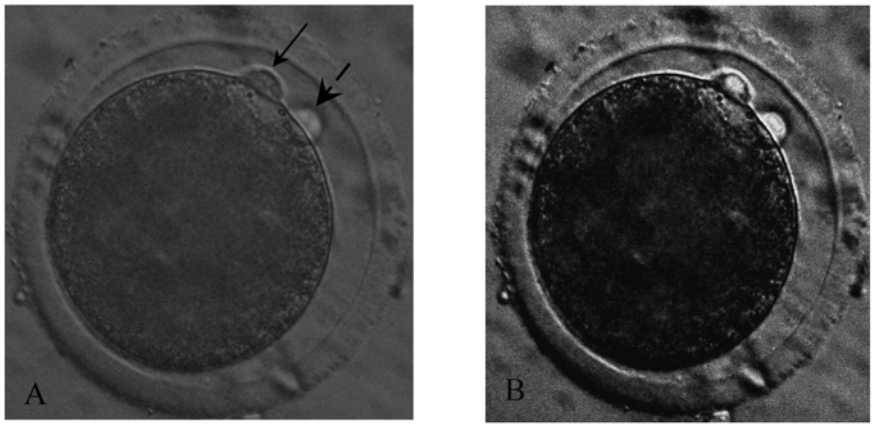

Fig. 1. – An oocyte with an extrusion cone following 0.5h of demecolcine treatment. Arrow shows the extrusion cones, and arrowhead indicates the first polar body (a). The condensed chromosome mass can be seen in the extrusion cones under fluorescent field (b) after Hoechst stain.

The oocytes with extrusion cone were successfully enucleated. As shown in Table 2, the bovine oocytes matured *in vitro* treated with 0.5μg/mL demecolcine were examined 0hr to 2.5hr, the ECR in group 2hr was still significantly higher than those in groups 0.5hr, 1hr and 2.5hr, individually ($P>0.05$). When we examined the effects of oocyte maturation time on the formation of extrusion cone, we found that the ECR of maturation 18hr was still significantly higher than those in groups maturation 14hr and 16hr, individually (Table 3). After 12-18hr maturation in vitro, COCs were further cultured for 2hr in the same solution supplemented by 0.5μg/mL demecolcine. The cumulus cells of all groups were removed at 20hr of culture by vortexing the COCs in 0.2% hyaluronidase in Ca^{2+}- and Mg^{2+}- free Dulbecco's PBS. The ECR in group maturating for 18hr was significantly higher than those in groups maturating 14hr and 16hr, individually (Table 4).

As shown in Table 5, the proportions of IVF that developed into blastocysts were not significantly different among groups using DM treatment.

TABLE 1

DM concentration influences ECR

DM (μg/mL)	No. of oocytes	ECR%	No. of reconstructed embryos	rate of cleavage%	rate of blastocyst%
0	67	0.04^a(3/67)	□	□	□
0.4	66	54.54^b(36/66)	32	78.13^a(25/32)	12.00^a(3/25)
0.5	84	61.90^c(52/84)	48	79.17^a(38/48)	13.16^a(5/38)
0.6	78	44.87^b(35/78)	33	78.79^a(26/33)	11.54^a(5/26)

Within columns, values with different superscripts differ significantly ($P<0.05$). "□"means no results. The following tables are the same.

TABLE 2

Influence of DM incubation time on ECR

Time (hr)	No. of oocytes	ECR%	No. of reconstructed embryos	rate of cleavage%	rate of blastocysts%
0	56	0.05[a] (3/56)	□	□	□
0.5	74	56.76[b](42/74)	40	82.50[a](33/40)	9.09[a](3/33)
1	49	67.34[b](33/49)	33	78.79[a](26/33)	7.69[a](2/26)
2	81	76.54[c](62/81)	61	77.05[a](47/61)	6.38 [a](3/47)
2.5	68	66.18[b](45/68)	43	76.74[a](33/43)	6.06 [a](2/33)

TABLE 3

Oocyte maturation time influence on ECR

Maturation time(hr)	No. of oocytes	ECR%	No. of reconstructed embryos	rate of cleavage%	rate of blastocysts%
14	18	11.11[a](2/18	7	14.29[a](1/7□	□
16	45	33.33[b](15/45)	14	50.00[b](7/14)	□
18	88	73.86c(65/88)	65	89.23[c](58/65)	13.79[a](8/58)
20	75	65.33[c](49/75)	48	81.25[c](39/48)	10.26[a](4/39)

TABLE 4

Granular cell existence and add DM influence on ECR

Maturation time(hr)	No. of oocytes	ECR%	No. of reconstructed embryos	rate of cleavage%	rate of blastocysts%
12	15	0.13[a](2/15)	□	□	□
14	42	42.86[b](18/42)	18	55.56[a](10/18)	□
16	86	73.26[c](63/86)	54	68.52[b](37/54)	13.33[a]5/37
18	73	80.8259/73	50	86.00[c](43/50)	18.60[a]8/43

TABLE 5

Effect of DM on embryonic development of and *in vitro* fertilized bovine oocytes

group	No. of oocytes	rate of cleavage %	rate of blastocysts %	Number of blastocyst cells
Control	95	83.16[a](79/95)	39.24[a](31/79)	117[a]
Add to DM	87	81.61[a](71/87)	36.62[a](26/71)	113[a]

DISCUSSION

This study provided encouraging results to efficiently assisted-enucleate bovine oocyte. The oocyte enucleation procedure is crucially important to cloning efficiency by eliminating any genetic contribution of the recipient cytoplasm, and for excluding the possibility of parthenogenesis (Dominko et al., 2000; Li et al., 2004). Traditionally, mammalian oocyte cytoplasts are prepared by physically removing nuclear chromatin by micromanipulation techniques in preparation to receive the donor genome (Wilmut et al., 1997; Cibelli et al., 1998). Enucleated oocytes arrested at MII are subsequently ''reconstructed'' by the addition of the donor karyoplast, typically using either electrofusion (Wilmut et al., 1997) or microinjection techniques (Wakayama et al., 1998).

Enucleated oocytes were stained with Hoechst and exposure to ultra violet may cause damages of cytoplasmic organelles and mitochondrial DNA, and result in low cloning efficiency (Bell et al., 1997; Dominko et al., 2000). Chemically assisted enucleation is probably a better procedure for animal cloning. Recently, with colcemid assisted enucleation, cloned rabbit fetus (Yin et al., 2002a), pigs (Yin et al., 2002b), and mice (Gasparrini et al., 2003) were produced. More than 70% of bovine oocytes treated with colcemid had a membrane bleb in which the condensed maternal chromosomes are located and are easily removed by aspiration. Enucleation rate is very high (96%), which insures that the maternal genetic

material is removed, and eliminates the possibility of the involvement of maternal genetic residual in NT embryos.

Demecolcine is a specific microtubule inhibitor that binds to tubulin dimers and prevents microtubule polymerization, thus resulting in the loss of the dynamic spindle microtubules. As previously demonstrated in the mouse, transient treatment of pre-activated oocytes with this tubulin-binding agent allows the enucleation of oocytes by the expulsion of the entire chromosome complement within the PBs (BAGUISI & OVERSTROM, 2000; IBANEZ et al., 2003). The removal of chromosomes from activated eggs at the telophase stage is also effective (IBANEZ et al., 2003), but decreased maturation promoting factor activity might decrease the viability of nuclear-transferred eggs with somatic cells (TANI et al., 2001). Matured pig eggs treated with demecolcine had a membrane extrusion cone in which the condensed chromosome mass was located. Similar to porcine oocyte by an actin-rich domain after DM treatment, bovine meiotic chromosomes are also observed within an extrusion cone. Although the mechanisms of action of demecolcine are not clear, the appearance of the extrusion cone might be related to the condensation of maternal chromosomes. When eggs were treated with demecolcine for 0.5; 1; 2 and 2.5hr, the proportions of eggs with condensed chromosomes were 56.76%, 67.34%, 76.54%, and 66.18%, respectively. Most of eggs with condensed chromosomes had an extrusion cones membrane. When oocytes were incubated without demecolcine, extrusion cones were present in only 0.05% of oocytes, and the size of these cones was considerably smaller than when the agents were used. Demecolcine incubation causes a significant increase in the number of oocytes with extrusion cones formation started at 0.5hr. All of these oocytes could be success-fully enucleated, and the reliability of enucleation was 100%. The DM assisted enucleation procedure made the NT work more rapid, because the only sophisticated instrument required, the inverted fluorescent microscope, was no longer required. According to our knowledge, the procedure described in this work is the most efficient and accurate published enucleation method for bovine oocytes. The high efficiency achieved in our experiments was not only the consequence of incubation with demecolcine, but was due to *in vitro* matured oocytes with granualar cell were added to DM. Demecolcine incubation without granualar cell also induced extrusion, but the proportion of oocytes with cones was small, and reached only 64%. However, a significant increase in the number of oocytes with extrusion cones was observed with granualar cell (73.86% vs 80.82%). The viablity of NT embryos produced by using without granualar cell of oocytes in DM incubation was generally lower than that of with granualar cell produced NT embryos (13.79% vs 18.60%). Previous work demonstrated that contact of the cumulus-enclosed oocyte with an intact, undisturbed granulosa layer provides the greatest continuity linking literature reports together. The granulosa surface and cell-to-cell interaction with the oocyte is potentially the important factor in meiotic regulation. (TSAFRIRI et al., 1976; CHANNING & TSAFRIRI, 1997), yet the same researchers obtained almost complete inhibition using co-culture of porcine oocytes and granulose monolayers (TSAFRIRI & CHANNING, 1975). Rat granulosa monolayers are reported to cause inhibition of rat oocytes but only after the granulosa cells have been cultured 24hr prior to the addition of oocytes (TSAFRIRI, 1978). This suggests the need for regeneration of cell surface receptors. The different molecular weights reported for OMI (TSAFRIRI et al.,1976; JAGIELLO et al.,1977; STONE et al.,1978) indicate that fragments of a larger molecule are appearing in fluids causing inhibition of oocyte maturation; such fragments perhaps derives from portions of cell surface constituents. OHNO & SMITH (1964) described the precocious maturation past the dictyotene stage and the ensuing degeneration of primary oocytes that fail to develop when into intimate contacts with follicle cells in foetal calf ovaries. This close association between the oocyte and surrounding follicle cells is continued throughout all stages of oocytic development until preovulatory changes occur (GILULA et al., 1978). A more unified approach to the regulation of oocyte meiosis is provided by viewing it as a problem concerning cell surface interactions or contacts. This is an approach that encompasses not just control in antral follicles but that extends back to the original contact between follicle cells and oocytes at the time of primordial follicle formation.

In conclusion, the present study shows that assisted enucleation can be accomplished in bovine oocytes using demecolcine.

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Seasonal and annual changes in the diet of the Red-backed Shrike *Lanius collurio* in farmland of Eastern Poland

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ABSTRACT. The aim of the study was to identify differences in diet composition of the Red-backed Shrike during the breeding period and between years related to habitat diversity of territories. The study was conducted in 1999-2003 in extensive farmland of Eastern Poland. The diet composition the Red-backed Shrike was analysed based on examination of animal remains in pellets found under nests and places where the birds often stayed. The material was divided into two groups: pellets collected in territories with predominantly arable land and others with predominantly grasslands (meadows and pastures). In territories with the prevalence of arable land the proportion of Hymenoptera in the Red-backed Shrike diet increased, and of Coleoptera decreased, with the progress of the season. A corresponding pattern was not observed in territories comprising grasslands. Territories of the Red-backed Shrike located in the same places in two years did not differ in the proportions of Coleoptera and Hymenoptera in a birds' diet. This can be an evidence of a food stability of Red-backed Shrike's territories in consecutive years and of dietary preferences of this species. Grasslands supported a greater abundance and variety of invertebrates in comparison with arable land. Thus, actual food preferences of Red-backed Shrikes in grassland territories could be causing the lack of a relation between season and the proportion of a given taxon in the diet. Thus, the occurrence of feeding preferences in sites of restricted feeding conditions may not entirely reflect actual food selectivity of the species.

KEY WORDS : Red-backed Shrike, *Lanius collurio*, food, extensive farmland

INTRODUCTION

The diet composition of the Red-backed Shrike *Lanius collurio* (Linnaeus, 1758) has been extensively described in literature (summaries in: CRAMP & PERRINS, 1993; LEFRANC & WORFOLK, 1997; HARRIS & FRANKLIN, 2000). The diet composition of the Red-backed Shrike can vary depending on many factors including geographical location or weather conditions that may in turn influence the activity of potential prey (CRAMP & PERRINS, 1993; TRYJANOWSKI et al., 2003a). Methods used for the analysis of diet (TRYJANOWSKI et al., 2003b), as well as the timing of collecting the material within a season (KARLSSON, 2004) may also affect the results of dietary studies.

The Red-backed Shrike is an endangered species with a decreasing trend in numbers in Europe, especially in its western part (FORNASARI et al., 1997). Thus, the knowledge of the diet and food preferences of this species is very important for its active protection (KUPER et al., 2000; TRYJANOWSKI et al., 2003a).

The aim of the present study was to characterise differences in the diet composition in the Red-backed Shrike during the breeding season and between years, with respect to habitat diversity in birds' territories. Observations were conducted in agricultural landscape of Eastern Poland, which is characterised by extensive farming practice. Such areas, because of the type of land use, are rare in Europe and differ even from the farmland of Western Poland. These differences are reflected in a trend of increasing numbers of birds observed in recent years in many species that inhabit this type of landscape, including the Red-backed Shrike (DOMBROWSKI et al., 2000; DOMBROWSKI & GOŁAWSKI, 2002).

MATERIALS AND METHODS

The study was conducted 10-15km NE from Siedlce (52º12'N, 22º17'E). The study area consisted of 855ha of farmland with low intensity of farming practice (little mineral fertilisers, herbicides) and considerable fragmentation of fields. Arable land predominated (53.5%), with mainly crops of rye and potatoes. Meadows and pastures covered 21.1%, and the proportion of fallows was 2.2%. Besides these open habitats, there were woodlands and orchards. The structure of land use did not change during the study.

The material was collected in June and July 1999-2003. The diet composition of the Red-backed Shrike was analysed based on examination of animal remains in pellets found under nests and places where the birds often stayed. In this paper, four orders of invertebrates were analysed - only these which were abundantly represented in birds' food (GOŁAWSKI, 2006a). Prey was classified by an entomologist into the lowest possible taxon. Numbers of invertebrates were determined according to the number of remains characteristic for a taxon, i.e. heads, legs, parts or whole coverts.

The material was divided into two groups: collected in territories with predominantly (over 70%) arable land and those with predominantly grasslands (meadows and pastures). The first group consisted of 18, and the second – 14 territories of the Red-backed Shrike. A circle of 70m radius with the centre in the nest location was assumed as

a Red-backed Shrike territory. The accepted territory size (1.5ha) is the average territory size in this species in Europe (CRAMP & PERRINS, 1993). To assess shrikes' diet, the numbers of territories was used as a sampling unit (KATZNER et al., 2005) to avoid pseudoreplication which occurs when the number of pellets is used. To minimize the impact of unequal sampling in different territories, I analysed only data from territories where more than 25 prey items were collected. These pellets were analysed at four half-month stages in June and July, similar to other studies (e.g. KARRLSON, 2004). In addition, the proportions of two most abundant orders of insects: Coleoptera and Hymenoptera (other orders of insects represented in lower numbers) in birds' food was analysed in the same territories in different seasons. In this case, pairs of data collected in corresponding half-month periods were compared.

In statistical tests Gamma correlation coefficient and Wilcoxon matched pairs test were used. These calculations were done with Statistica 6.0 (STATSOFT, 2003).

RESULTS

Coleoptera predominated in the diet of Red-backed Shrikes which inhabited territories comprising arable land (n=18 territories; Coleoptera 81.4%±17.59; mean±SD), followed by Hymenoptera (9.0%±7.18), Heteroptera (8.2%±16.43) and Orthoptera (2.2%±1.11). The dominance structure of prey was similar in the diet of birds from territories including meadows and pastures (n=14 territories), and the dominants were Coleoptera (77.3%±22.60), followed by Hymenoptera (13.8%±10.20), Heteroptera (2.6%±1.32) and Orthoptera (2.6%±1.85). The representation and rank of importance of these invertebrates in birds' food was similar in all analysed periods (Tables 1 & 2).

In territories with arable land an increase of proportion of Hymenoptera (r Gamma=0.34, p=0.025) and a decrease of the proportion of Coleoptera (r Gamma=-0.29, p=0.012) with the progress of the season was observed. For the two remaining orders of invertebrates, no significant trends were observed (Table 1). In territories which included grasslands, tendencies in changes of proportions of Coleoptera and Hymenoptera in birds' food were similar (Table 2), but not statistically significant (p>0.103 in both cases). For Heteroptera and Orthoptera, similar to the situation in territories with arable fields, no seasonal changes in their proportions in the diet could be detected (Table 2).

The analysis of the composition of the Red-backed Shrike diet in the same territories occupied in two seasons did not reveal any differences in the percentage of both Coleoptera (Wilcoxon matched pairs test, z=0.36, p=0.722, n=11) and Hymenoptera (Wilcoxon matched pairs test z=0.65, p=0.515, n=9). Thus I assume that these two orders of invertebrates had similar proportions in the diet in the same territories during consecutive seasons (Fig. 1).

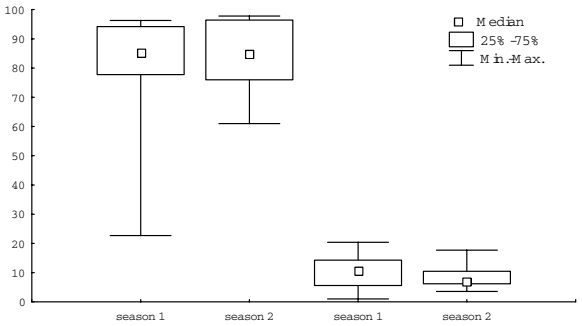

Fig. 1. – Proportions of Coleoptera (left bars, n=11 territories) and Hymenoptera (right bars, n=9 territories) in the diet of the Red-backed Shrike during two seasons.

TABLE 1

Proportions ± SD (n territories) of four of the most abundant taxa in the diet of the Red-backed Shrike in territories comprising arable land in farmland of Eastern Poland

Taxa	1-15 June	16-30 June	1-15 July	16-31 July
Coleoptera	85.0±12.62 (7)	85.6±8.31 (10)	80.8±11.79 (9)	68.1±26.64 (5)
Hymenoptera	10.2±11.27 (6)	7.7±5.26 (8)	9.3±5.35 (8)	15.3±6.42 (4)
Heteroptera	1.5±0.99 (2)	5.3±2.34 (6)	5.2±0.28 (2)	27.6±38.11 (2)
Orthoptera	1.5±0.99 (2)	3.7±2.27 (5)	5.2±0.28 (2)	2.3 (1)

TABLE 2

Proportions ± SD (n territories) of four of the most abundant taxa in the diet of the Red-Backed Shrike in territories comprising grasslands in farmland of Eastern Poland

Taxa	1-15 June	16-30 June	1-15 July	16-31 July
Coleoptera	86.8±8.32 (6)	77.6±9.25 (3)	80.5±14.12 (7)	69.7±4.24 (2)
Hymenoptera	12.3±8.28 (4)	15.7±7.50 (3)	14.3±13.58 (7)	21.7±0.71 (2)
Heteroptera	3.1±1.56 (2)	3.7±1.86 (3)	2.6±2.02 (6)	3.7 (1)
Orthoptera	10.1±11.46 (2)	3.4±0.92 (2)	1.2±0.10 (3)	4.9±1.70 (2)

DISCUSSION

The dominance structure of insect orders in the Red-backed Shrike diet, based on analysis of pellets, did not depart from proportions of these orders in the diet of these birds observed in other places in Europe (MANN, 1983; HERNÁNDEZ et al., 1993; WAGNER, 1993; OLSSON, 1995; KUPER et al., 2000; TRYJANOWSKI et al., 2003b; KARLSSON, 2004; GOŁAWSKI, 2006a). Proportions of two dominant orders of insects did not differ between territories located in the same places in consecutive years. This may be evidence of food preferences of the Red-backed Shrike, as it was demonstrated previously that this species prefers insects from these two orders (HERNÁNDEZ et al., 1993; GOŁAWSKI, 2006b). Moreover, this fact may prove food stability of these territories between years. This is probably an effect of the extensive use of farmland in this part of Poland (low use of mineral fertilisers and herbicides, mosaics of habitats, USW, 2005), and a lack of major changes in the structure of land use.

In territories comprising arable land, changes in food composition during the season were found. The proportion of Coleoptera in Red-back Shrike diet decreased, while the proportion of Hymenoptera increased during the breeding season. This pattern corresponded with results obtained in Finland (KARLSSON, 2004). However, a distinct increase in the proportion of Orthoptera in the diet with the progress of the season was observed in Finnish birds (KARLSSON, 2004). In the present study, although similar tendencies of seasonal changes in diet were observed in territories located on grasslands, these were statistically not significant. This difference could potentially be due to low food abundance in arable land in comparison with grassland (GOŁAWSKI, 2005 msc). Thus, in arable land Red-backed Shrikes have limited possibilities of catching suitable prey and depend upon food available in this habitat. In contrast, grasslands, which support greater abundance and diversity of animals, offer a richer food base allowing shrikes to actively select suitable prey (in the described area this is Coleoptera and Hymenoptera), and avoid other insects (e.g. Orthoptera; GOŁAWSKI, 2006b). Thus, in grassland selection of prey is less dependent on changes in numbers of insects (species) during the season. The different height of herbaceous vegetation in territories comprising arable land and grasslands can have some influence on the diet composition of the Red-backed Shrike, by affecting the way birds hunt. With the progress of the season crops grow taller, making potential prey less visible and forcing Red-backed Shrikes to catch most of their prey in flight. Territories in grasslands have lower vegetation (because of hay mowing, cattle grazing) and in such conditions Red-backed Shrikes can hunt on the ground. Indeed, in Hungary, Red-backed Shrikes more often catch prey in flight in territories with tall herbs in comparison with territories of low vegetation (MOSKÁT, 2001).

The obtained results indicate the importance of grassland for the ability of the Red-backed Shrike to collect suitable food. The importance of grasslands was confirmed by the studies on the breeding performance of the Red-backed Shrike in the studied region; the number of fledglings in clutches of these shrikes was positively correlated with the area of meadows and pastures in their territories (GOŁAWSKI & MEISSNER, 2007). Changes in land use in Europe lead to the decrease of grassland and the increase of arable land coverage (FULLER et al., 1991). The same trend of change occurred also in the studied area after the accession of Poland to the European Union (subsidies for farmers for land cultivation). Besides the decrease of grassland area, the coverage of fallows also decreased, which has a positive effect on the number of fledglings in the Red-backed Shrike (GOŁAWSKI & MEISSNER, 2007). However, to protect this, as well as several other species (as e.g. Hoopoe *Upupa epops*, Great Grey Shrike *Lanius excubitor*), grassland areas as large as possible should be retained, especially pastures with low vegetation where collecting suitable food is easier for birds. Turning grassland into arable fields, often associated with cutting clumps of trees and bushes (simplification of the landscape) would undoubtedly lead to the decrease in numbers of the Red-backed Shrike in Poland, where one of its greatest populations in Europe still breeds (BIRDLIFE INTERNATIONAL, 2004).

The structure of vegetation in a territory and its food abundance can influence the diet composition, and thus the interpretation of results pertaining to food preferences. The present results confirm that it is possible to find a relation between the available prey and prey used by shrikes in sites with relatively low abundance and diversity of animals (KUPER et al., 2000). This may be so because such relation is easier to detect in places poorer in food. However, dietary preferences found in such conditions may not completely reflect the actual food selectivity of a species.

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Length-weigth relationships for syngnathid fishes of the Aegean Sea, Turkey

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ABSTRACT. In this paper we present length–weight relationships of three pipefishes, *Syngnathus acus*, *Syngnathus typhle Nerophis ophidion,* and two seahorse species, *Hippocampus hippocampus, Hippocampus guttulatus* from İzmir Bay, on the Turkish coasts of the Aegean Sea. Overall, 1010 specimens of five different species of Syngnathidae were weighed and measured. The sample size ranged from 29 for *H. hippocampus* to 570 for *S. acus*. The values of the exponent *b* in the length–weight regressions ($W=aL^b$) varied between 2.42 (*N. ophidion*) and 3.54 (*S. acus*). Linear regressions of length–weight relationships were significant for all species. Positive allometry in weight vs. length for *S. acus*, isometry in *S. typhle* and *H. hippocampus* and negative allometry for *H. guttulatus* and *N. ophidion* were observed.

KEY WORDS : Length–Weight Relationship, Pipefish, Seahorse, Aegean Sea, Turkey

INTRODUCTION

Estuaries are nursery and over-wintering areas for many marine fish species (Beyst et al., 1999). Fishes of the Sygnathidae are typically small and cryptic and are associated with vegetated and epibentic habitats (Pollard, 1984; Kuiter, 2000). Syngnathids are among the most abundant groups in seagrass-associated fish communities of the benthic habitats (Pollard, 1984). In terms of a community dominance index, syngnathids were the highest ranked family for both the Atlantic-Mediterranean and Indo-Pacific region in benthic habitats (Howard & Koehn, 1985; Vitturi et al., 1998). The Mediterranean and Western Atlantic species migrate seasonally, moving into shallow, vegetated areas during spring and remaining there until the late fall when migration back to the deeper channel areas occurs (Mercer, 1973; Dawson, 1982). However, a small numbers of individuals may overwinter in seagrass beds (Dawson, 1982). The genus *Syngnathus* is represented by nine species in the Mediterranean (Dawson, 1982; Bilecenoglu et al., 2002) and six species in the Aegean Sea (Bilecenoglu et al., 2002). The genus *Hippocampus* is represented by two species in Aegean Sea (Whitehead et al., 1986; Bilecenoglu et al., 2002). A review of the current literature indicates that little is known about biology and growth rate of pipefish and seahorses in the Mediterranean basin.

Length-weight relationships of fish, in general, are important because they: (a) allow an estimate of the condition of fish; (b) allow the estimation of biomass from length observations; (c) allow the conversion of growth-in-length equations to growth-in-weight; and (d) are useful for between-region comparisons of life histories of species (Pauly, 1993; Gonçalves et al., 1996; Binohlan & Pauly, 1998). Length-weight relationships are also originally used to provide information on the condition of fish and may help determine whether somatic growth is isometric or allometric (Ricker, 1975).

Information about length-weight relationships for the pipefish and seahorse species from Aegean sea is scarce and incomplete (Koutrakis & Tsikliras, 2003; Valle et al., 2003; Lamprakis et al., 2003; Verdiell-Cubedo et al., 2006). Regarding Turkish seas, only two studies focus on syngnathid fishes, dealing with aspects of diversity and abundance of the family Syngnathidae from Erdek Bay, the Sea of Marmara (Keskin et al., 2002) and the ecomorphology of the pipefish (Familia: Syngnathidae) distributed in the Camalti Lagoon, İzmir Bay (Gurkan, 2004). Studies on length-weight relationships for the Turkish seas are mainly related to fish having an economic importance. A single document by Ozaydin & Taşkavak (2006), who studied length-weight relationships for 47 fish species from İzmir Bay, gives any information on length-weight relationships for three species of pipefish, *Syngnathus acus, S. typhle* and *Nerophis ophidion*.

We, here, report length–weight relationships for three pipefish and two seahorse species from İzmir Bay in the eastern Aegean Sea.

MATERIALS AND METHODS

Between 2000 and 2002, the pipefish species, *Syngnathus acus*, *Syngnathus typhle* and *Nerophis ophidion,* were obtained monthly with trammel nets (mesh sizes-bar lengths: 22, 24, 26 and 28mm) in the Camalti Lagoon, İzmir Bay (Aegean Sea) (Fig. 1). Specimens of two seahorse species, *Hippocampus hippocampus* and *Hippocampus guttulatus* were obtained from commercial fishermen on February 2000. *H. hippocampus* and *H. guttulatus* specimens were based on a single sampling since this type of fishing (beach seine) was prohibited along the Turkish coasts thereafter. Lengths (TL – total length – for pipefish and SL – standard length – for seahorse) were measured to the nearest mm and animals were weighed (W) to the nearest g. Standard length of seahorse is expressed as sum of head length, trunk length and tail length, using a curved measurement of trunk length from the midcleithral ring to the last trunk ring (Lourie et al., 1999).

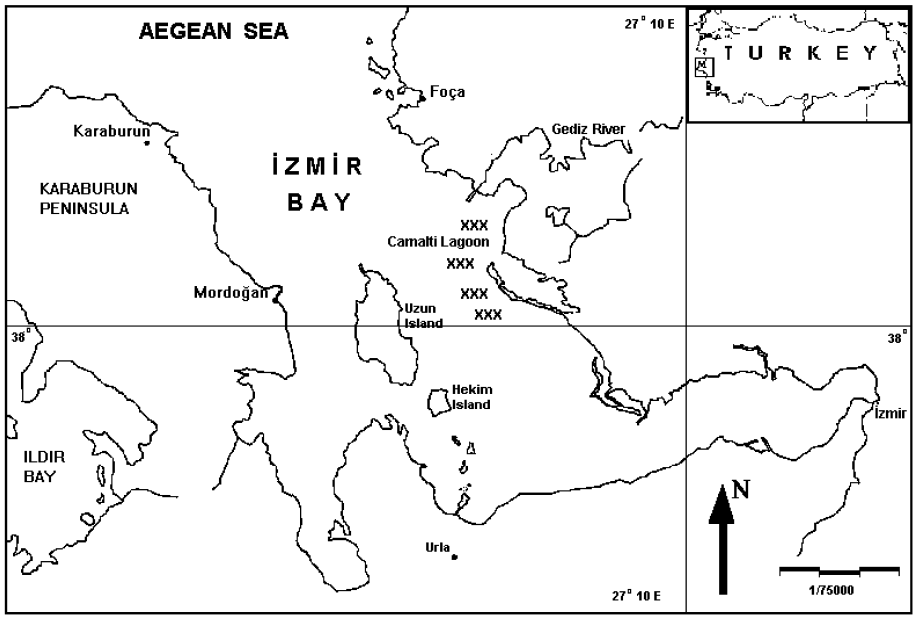

Fig. 1. – Map showing the location where sampling was carried out.

Length to weight relationship for total body weight was calculated using the equation $W=aL^b$, where W is weight (expressed in grams), L is length (TL and SL, expressed in mm), a is the intercept, and b is the slope. The degree of association between the variables was computed by the determination coefficient, r^2. The parameters a and b were estimated by linear regression on the Log-transformed (Log_{10}) equation $log(W)=log\ (a)+\ b\ log\ (L)$. The significance of the regression was assessed by ANOVA, and the b-value for each species was tested by t-test to verify that it was significantly different from the predictions for isometric growth (b=3).

RESULTS AND DISCUSSIONS

Members of the Family Syngnathidae consistently form an important component of the ichthyofauna of vegetated estuarine habitats and are among the most abundant groups in this type of habitat (POLLARD, 1984). Although the fishes of the family Syngnathidae are not economically important, they are significant from the aspect of ichthyofauna conservation and overall fish diversity (CAKIĆ et al., 2002).

Overall, 1010 specimens of five different species of Syngnathidae were weighed and measured to estimate length-weight relationships. The sample size ranged from 29 for *Hippocampus hippocampus* to 570 for *Syngnathus acus*. During the course of the study, *S. acus* was the most abundant one. Length-weight relationships of three pipefish and two seahorse species examined for the İzmir Bay (Aegean Sea) are given in Table 1. Relationships (linear regressions) were significant for all species (P<0.001), with r^2 values being greater than 0.95 for *S. acus* and *S. typhle*; and about 0.75 for *Nerophis ophidion* and *H. hippocampus*; the single exception was *H. guttulatus* (r^2=0.64 and P<0.05).

Values of b equal to 3 indicate that the fish grows isometrically; values different from 3 indicate allometric growth. The exponent b varied between 2.42 for *N. ophidion* and 3.54 for *S. acus* (Table 1). An over-proportional increase in length relative to growth in weight is reflected in an exponent of b<2.5 or to the contrary, an exponent of b>3.5 indicates an over-proportional increase in weight relative to growth in length (FROESE, 2006).

TABLE 1

Descriptive statistics and estimated parameters of the length–weight relationship for five species collected from the bay of İzmir, Aegean coast of Turkey (N: sample size, S.E.: standard error, Range: minimum and maximum, a and b: parameters of the length–weight relationship, r^2: coefficient of determination, Mean L: Mean Length in mm, Mean W: Mean Weight in g., and TL: Total length in mm.

Species	N	Length Characteristics		Weight Characteristics		$W=aL^b$		
		Mean L±SE	Range	Mean W±SE	Range	a	b±95%C.I	r^2
S. acus	570	100.98±1.09	33-256	0.60±0.04	0.01-12.29	6E-08	3.54±0.03	0.95
S. typhle	125	155.37±3.74	40-258	1.49±0.12	0.01-8.2	3E-07	3.00±0.06	0.96
N. ophidion	86	145.78±3.04	78-214	0.35±0.02	0.06-0.83	3E-06	2.42±0.16	0.74
H. hippocampus	29	113.21±0.09	80-140	3.94±0.01	0.95-6.55	0.001	3.14±0.34	0.76
H. guttulatus	200	133.33±0.09	100-165	6.54±0.01	2.54-111.88	0.010	2.47±0.13	0.64

Concerning growth types, the length-weight relationships revealed that weight increases isometrically with length for *S. typhle* (t_{cal}=0.01<$t_{0.05(124)}$=1.98) and *H. hippocampus* (t_{cal}=0.41<$t_{0.05(28)}$=2.05). Positive allometry in *S. acus* (t_{cal}=18.00>$t_{0.05(569)}$=1.96) and negative allometry in *N. ophidion* (t_{cal}=2.80>$t_{0.05(85)}$=1.99) and *H. guttulatus* (t_{cal}=4.05>$t_{0.05(199)}$=1.98) were observed. CARLANDER (1977) demonstrated that values of b<2.5 or >3.5 are often the consequence of small sample sizes. Given the sample sizes in our study this is unlikely to be the case. However, these variations from isometry may be due to the very small specimens included in the regression that had not yet reached adult body size.

Other studies conducted in Turkish waters (GURKAN, 2004; OZAYDIN & TAŞKAVAK, 2006) reported values of the scaling exponent ranging from 2.36 to 3.43 and from 2.13 to 3.63 for three pipefish species (*Syngnathus acus, S. typhle* and *Nerophis ophidion*). Our results are quite similar to those reported by GURKAN (2004) and OZAYDIN & TAŞKAVAK (2006), i.e., positive allometry in *S. acus* and negative allometry in *N. ophidion*, in spite of limited sample sizes in previous studies.

The values of the scaling exponent b for three pipefishes (Table 2) ranged from 2.13 (*N. ophidion*) to 3.73 (*S. acus*) and our results remained within the ranges given. Our results mostly agreed with the pipefish studies given in Table 2. Mean condition of specimens as well as the difference in condition between small and large specimens vary between seasons, localities and years, resulting in different length-weight relationships (FROESE, 2006). Except for data reported by VALLE et al. (2003), who measured the standard length, most of these results in Table 2 revealed that weight increases isometrically with length for *S. typhle* but allometrically for *S. acus* and *N. ophidion*, as was observed in this study.

TABLE 2

Summary of the available studies on length–weight relationship for *Syngnathus acus*, *Syngnathus typhle* and *Nerophis ophidion* in various seas (N: sample size, a and b: parameters of the length–weight relationship, r^2: coefficient of determination).

Study	Locality	N	a	b	r^2
		Syngnathus acus			
COULL et al. (1989)	Moray firth	4	0.0006	3.53	–
KOUTRAKIS & TSIKLIRAS (2003)	Greece	5	0.0001	3.73	0.96
VALLE et al. (2003)	Spain	225	0.0007	2.88	0.96
GURKAN (2004)	İzmir Bay	310	0.0001	3.43	0.89
OZAYDIN & TAŞKAVAK (2006)	İzmir Bay	202	0.0001	3.63	0.97
This Study	İzmir Bay	570	0.0001	3.43	0.91
MEAN			**0.0003**	**3.44**	
		Syngnathus typhle			
WORTHMANN (1975)	Kiel Bigth	2	0.0003	2.94	–
VALLE et al. (2003)	Spain	167	0.0002	3.17	0.96
GURKAN (2004)	İzmir Bay	93	0.0001	3.05	0.91
OZAYDIN & TAŞKAVAK (2006)	İzmir Bay	14	0.0002	3.22	0.94
This Study	İzmir Bay	125	0.0001	3.00	0.96
MEAN			**0.0002**	**3.08**	
		Nerophis ophidion			
WORTHMANN (1975)	Kiel Bigth	1	0.0002	2.72	–
GURKAN (2004)	İzmir Bay	68	0.0001	2.36	0.74
OZAYDIN & TAŞKAVAK (2006)	İzmir Bay	11	0.0009	2.13	0.82
This Study	İzmir Bay	86	0.0001	2.42	0.74
MEAN			**0.0003**	**2.41**	

No data of length-weight relationship is available for *Hippocampus hippocampus*. When we compare our result (b=2.47) with those given by VERDIELL-CUBEDO et al. (2006; b=2.91), there is a significant difference between the length-weight relationships for *H. guttulatus* of Mar Menor coastal lagoon (south-eastern Spain) and İzmir Bay (western Turkey). Length ranges given by VERDIELL-CUBEDO et al. (2006) for *H. guttulatus* were between 42 and 73mm, while they varied between 100 and 165mm in this study. Since the length ranges in both studies (VERDIELL-CUBEDO et al. 2006; and present study), do not overlap, the results are not comparable. For *H. hippocampus* and *H. guttulatus* no length-weight relationship information was available in FishBase (FROESE & PAULY, 2004). However, both Mediterranean seahorse species are listed as data deficient (DD; ver. 3.1, IUCN, 2001) indicating that more information is required. This listing also acknowledges that future research may show that another classification is appropriate making data on seahorses in general of great importance.

The length-weight relationship in fishes can be affected by a number of factors including season, habitat, gonad maturity, sex, diet and stomach fullness, health and preservation techniques and differences in the length ranges of the specimen caught (TESH, 1971; WOOTTON, 1998), which were not accounted for in the present study. Thus,

differences in length-weight relationships between this and other studies could potentially be attributed to the combination of one or more of the factors given above.

The members of the family Syngnathidae are among the most common species of shallow waters. The information gained in the present survey may enable fish biologists to derive weight estimates for the İzmir Bay syngnathids that are measured but not weighed. Consequently, the data presented here could serve for comparison with similar studies of bays, estuaries, and coastal lagoons of the Mediterranean, and could be of use when the pipefish and seahorse populations are subjected to commercial fishing, part of recovery programs, or other management and conservation activities.

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Cirrifera genitoductus sp.n. (Platyhelminthes, Proseriata, Coelogynoporidae) from the Belgian coast, with observations on its ultrastructure and its significance for the taxonomy of the Coelogynoporidae

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ABSTRACT. *Cirrifera genitoductus* sp.n. (Platyhelminthes, Proseriata, Coelogynoporidae) is described from sandy beaches at the Belgian and Northern French coasts. The species is characterised by a cirrus with small spines, all of the same size, a prostate vesicle far behind the copulatory bulb, a long genito-intestinal duct and a pair of large gland complexes behind the genital pore. An identification key for the *Cirrifera*-species is given as are some data on the ultrastructure. A brief discussion on the character distribution within the Coelogynoporidae shows that the discovery of a species of *Cirrifera* with a genito-intestinal duct makes the demarcation of the genera even more blurred than it already is.

KEY WORDS: Platyhelminthes, Proseriata, Coelogynoporidae, *Cirrifera genitoductus,* ultrastructure, taxonomy.

INTRODUCTION

The family Coelogynoporidae was erected by BRESSLAU (1933) to include two proseriate species described by STEINBÖCK in 1924, *Coelogynopora gynocotyla* and *C. bresslaui*, in which there is a communication between the female system and the gut; hence the name of the genus. More than 20 species have been added to the genus since and several genera have been added to the family: *Vannuccia* Marcus, 1948, *Carenscoilia* Sopott, 1972, *Cirrifera* Sopott, 1972, *Invenusta* Sopott-Ehlers, 1976, *Ezona* Tajika, 1980, *Macroatrium* Riser, 1981, *Pseudovannucia* Faubel & Rohde, 1998 and *Stilivannucia* Faubel & Rohde, 1998. However, the more species become known, the greater the variation within each genus appears and the "boundaries" between the "genera" become more confused. The "mosaic-like" distribution of the genito-intestinal connection and some other characters within the Coelogynoporidae are discussed at the end of this contribution. An identification key for the *Cirrifera* species is given as well.

Thus far, one of the diagnostic characters for the taxon *Cirrifera* was the absence of a bursal organ and of a genito-intestinal connection. Now a species, *C. genitoductus* n.sp., is found that shows all other characters of the representatives of the taxon *Cirrifera*, but has a genito-intestinal duct. The species is described in detail, including a number of electron microscopic observations, adding some data to what was known of the ultrastructure of *Cirrifera aculeata* Ax, 1951 from the work of MARTENS & SCHOCKAERT (1985).

MATERIALS AND METHODS

Specimens were collected from sandy beaches using the $MgCl_2$-decantation method (see MARTENS, 1984), studied alive and mounted with lactophenol, one of those designated holotype. Specimens for transmission electron microscopy were fixed with 2% glutaraldehyde in 0.1M phosphate buffer and post-fixed in 1% OsO_4 in the same buffer at 4°C for 1h, dehydrated in a graded acetone series and embedded in araldyte using propylene oxide. Specimens were sectioned serially in alternating 1µm and ultrathin sections. Ultrathin sections were treated with aqueous solutions of 2% uranyl acetate and 1.2% lead citrate; 1µm sections were stained with toluidine blue (for further details: see MARTENS & SCHOCKAERT, 1985).

The holotype is deposited in the collections of the Swedish Museum of Natural History (Holotype Nr. 6339); all other material is deposited in the collection of the Research Group "Biodiversity, Phylogeny and Population Studies" of the Centre for Evironmental Sciences, Hasselt University, Diepenbeek, Belgium.

RESULTS

Family Coelogynoporidae Bresslau, 1933

Genus *Cirrifera* Sopott, 1972

Cirrifera genitoductus n. sp.

Localities

Mariakerke (Belgium): March, June and August 1983 (type locality); medium fine sand with fine shell debris in the mid-littoral. Heist (Belgium): October and November 1984

and in January and March 1984; fine sand, rich in silt in the mid-littoral. Wimereux (France): medium fine sand of the mid-littoral with abundant *Arenicola marina*: May 2006.

Etymology

The name refers to the presence of a genito-intestinal duct.

Description

Living adult animals (Fig. 1) are up to 16mm long and about 0.6mm wide. Like most coelogynoporids they have the tendency to curl up the posterior part of their body. The slender anterior tip is provided with two to three tufts of sensory hairs on either side. Anterior to the encapsulated brain there is a statocyst with four statocytes. The pharynx, directed ventrally, is about at 2/3 of the body. The intestine extends to a point close to the brain and has a solid pre-cerebral diverticulum reaching almost to the front end.

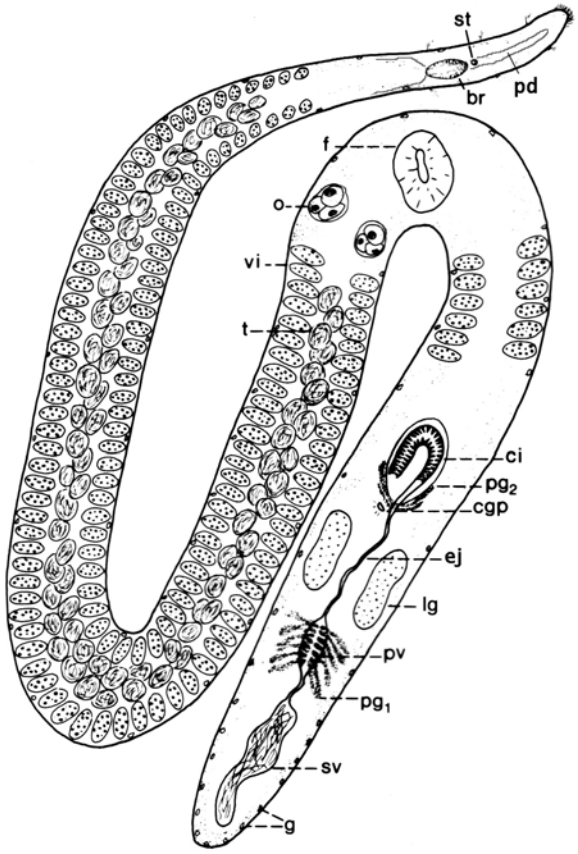

Fig. 1. – *Cirrifera genitoductus* n. sp.. Drawing of the living animal.

The epidermis (Fig. 2A,B) is 2.3 to 2.8µm thick and consists of irregularly-shaped cells with intra-epithelial lobate nuclei. The cilia are 6-8µm at the ventral side and 4-5µm at the dorsal side. Between the cilia are microvilli, about 0.05µm wide and 0.9µm long. The cilia have a long anteriorly-directed rootlet, almost parallel to the cell surface and a short rootlet perpendicular to it. The rostral rootlets converge into an extension of the anterior cell wall. A layer of ultrarhabdites can be observed just under the free cell surface. Various kinds of glands open through the epidermis among which are large glands, about 5µm in diameter with large electron-lucent granules up to 2µm in diameter. In the living animal, these glands are very apparent and might be confused with paracnids, which are absent in this species. Monociliary sensory collar receptors like those described e.g. by EHLERS & EHLERS (1977) were seen (but also receptors with two, three and four cilia) as were adhesive duo-gland organs.

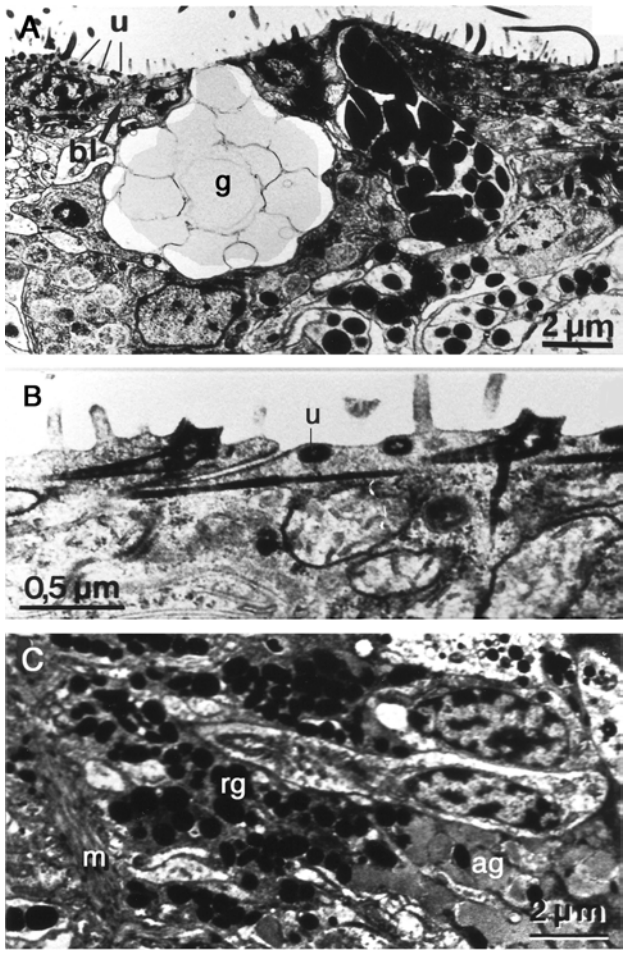

Fig. 2. – *Cirrifera genitoductus* n. sp.. Electronmicrographs of epithelial elements. A: Part of the epidermis showing some glands, among which the large hyaline glands, characteristic for species of *Cirrifera*. B: Longitudinal section through the epidermis, showing the ciliary rootlets and the ultrarhabdites. C: Section through the lateral glands.

The common genital pore without an obvious sphincter is about mid-way between pharynx and caudal end. The common genital atrium is almost completely filled with the protruding copulatory organ and is lined with a very low epithelium (Fig. 3A).

Behind the copulatory bulb there are two lateral masses of densely packed glands, very obvious in the living animal, even under the stereomicroscope (lg in Fig. 1). These glands discharge through the ventral epidermis and the gland cells contain round granules with low electron den-

sity (0.8µm in diameter) or very dense ovoid granules (0.2 to 1µm in diameter) (Fig. 2C).

The male genital organs consist of a row of up to 80 testis follicles, more or less in pairs, from some distance behind the brain to some distance in front of the pharynx. The follicle size increases from about 15µm in diameter anteriorly to about 50µm caudally (Fig. 1). The vasa deferentia fuse behind the copulatory organ and continue as a single seminal duct, which enters a large seminal vesicle at its anterior end (Fig. 3B, E). The long ejaculatory duct from the seminal vesicle to the copulatory organ enlarges to a prostate vesicle close after it has left the seminal vesicle. A second set of prostate glands enter the ejaculatory duct just before it enters the copulatory organ. The copulatory organ proper is a cirrus enclosed in a cirrus bulb (the duplex type of KARLING, 1956) (Fig. 3A,D). The ejaculatory duct enters the cirrus bulb at its caudal end, the cirrus bends over almost 180° and enters the atrium at its anterior side. Some protractor and dilatator muscles cross the bulb. The cirrus is about 130µm long, armed with up to 200 spines. These spines are symmetrically arranged in the cirrus, are 6µm long proximally and 8-9µm distally, with a basis 3-5µm wide. Cirrus and the bulb in which it is enclosed are surrounded by two muscle layers.

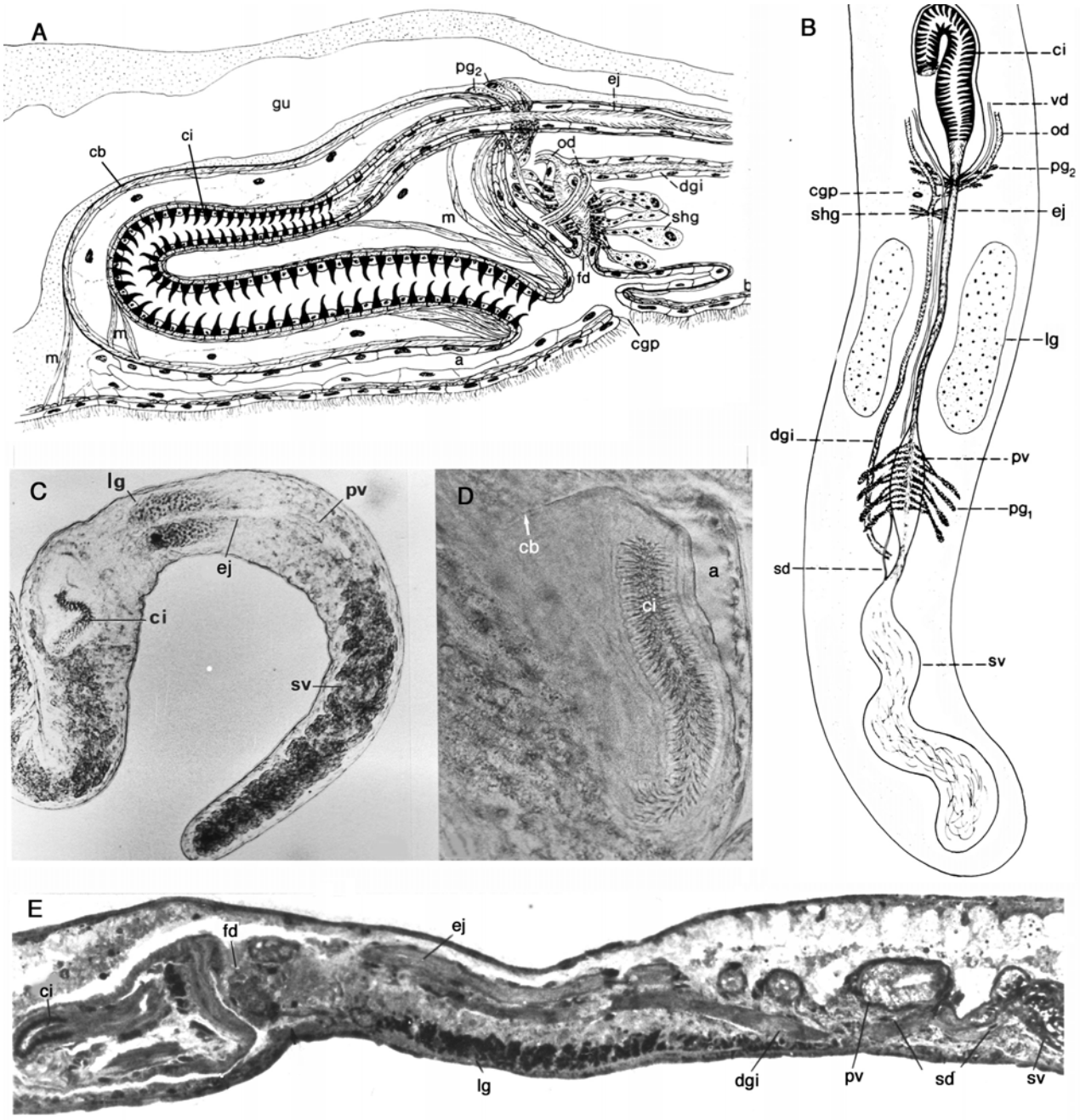

Fig. 3. – *Cirrifera genitoductus* n. sp.. Parts of the genital organs at the light microscopic level. A: Reconstruction of the atrial organs (seen from the left). B: Drawing of the post-pharyngeal parts of the genital system in the living animal. C: Micrograph of the same. D: Micrograph of the cirrus in the living animal. E: Micrograph of parts of the genital organs in a lateral longitudinal section.

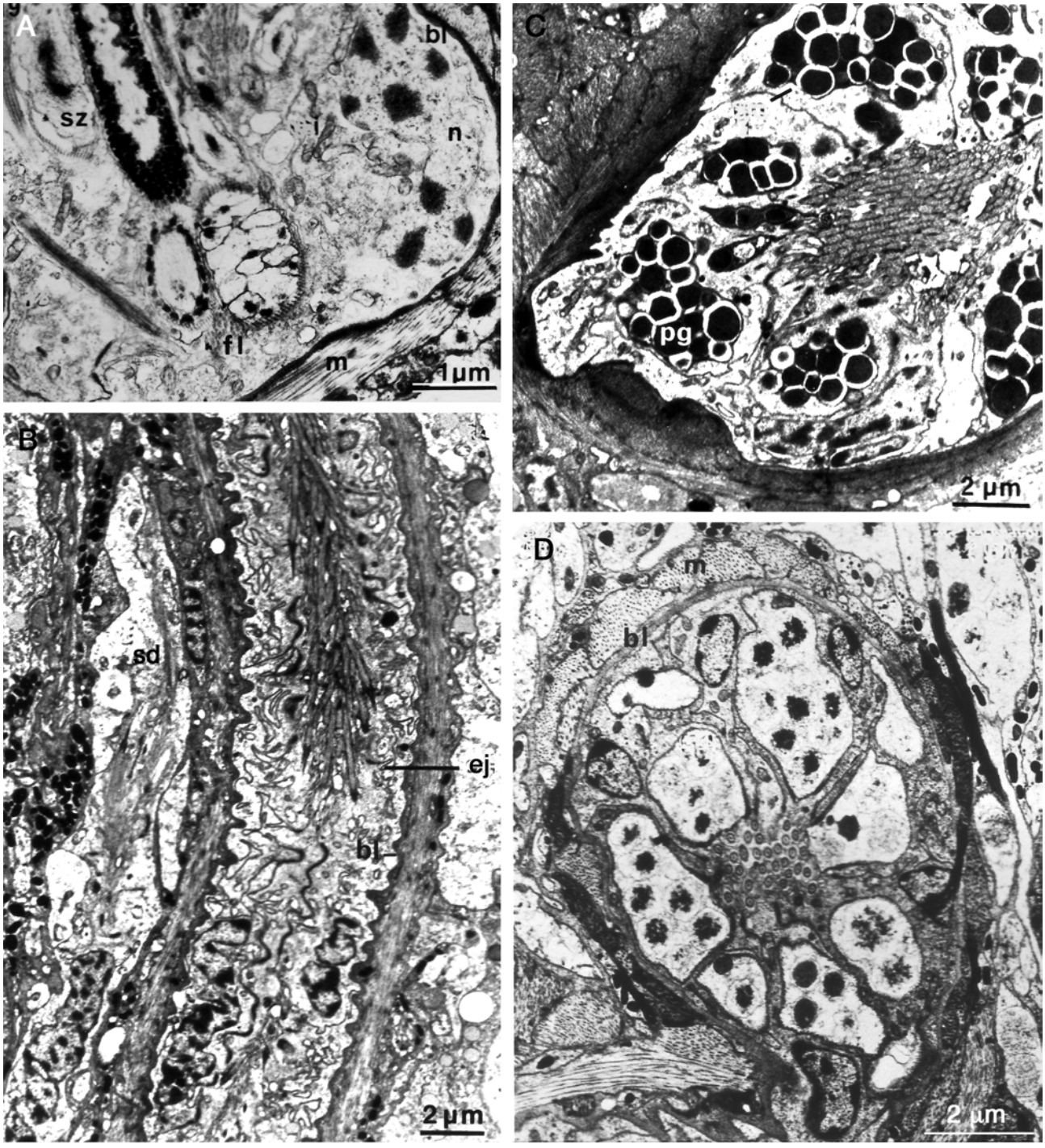

Fig. 4. – *Cirrifera genitoductus* n. sp.. Electronmicrographs of parts of the male genital system. A: The wall of the seminal vesicle, showing a spematozoon embedded in the epithelium. B: Longitudinal section though the ejaculatory duct. Note the convoluted plasmalemma of the epithelial cells, the thick basement membrane and the thick muscle layer. At the left is a section through the seminal duct with its very thin basement membrane. C: Section through the prostate vesicle, showing the glands with granules with a "halo". Note the thick muscle wall. D: Section through the prostate glands at the entrance to the copulatory organ, showing the granules with a flocculent core.

At the ultrastructural level, the male system is very similar to that in *Cirrifera aculeata* (see MARTENS & SCHOCKAERT, 1985). The two vasa deferentia, about 3µm in diameter, are lined with a ciliated epithelium as is the seminal duct. The circumference of the vasa deferentia consists of only two cells in any given cross-section, and of four cells in the seminal duct. The epithelium of the seminal vesicle is flattened with very few cilia. The basement lamina is very thin all along (0.15µm) with some dispersed muscles underneath, slightly thicker and more

numerous around the seminal vesicle. As in *C. aculeata*, spermatozoa are seen deeply embedded in the epithelial cells of the seminal vesicle (Fig. 4A). In the ejaculatory duct (Fig. 4B), which is 6-7µm wide, the basement lamina is almost twice as thick (0.25µm) and there is a strong muscle layer with spirally- but mainly longitudinally-running muscles. The epithelial cells have lobate nuclei and highly convoluted walls and are densely ciliated. The glands that enter the prostate vesicle (pg1) are filled with homogeneous electron-dense granules of 0.50-0.85µm in diameter with a "halo" (perhaps an artefact) (Fig. 4C). The prostate vesicle is surrounded by several muscle layers. The second set of prostate glands (pg2) produce membrane-bound granules, about 0.75µm in diameter, with a flocculent electron-dense centre and a fine granular, much less electron-dense peripheral material (Fig. 4D). Where the ejaculatory duct becomes cirrus, the cilia disappear and the epithelium cells have 1µm long microvilli. More distally, as in *C. aculeata*, each cell contains a spine that protrudes from the cell (Fig. 5A,B). The protruding part of a spine (*in Fig. 5B) has an homogeneous, very electron-dense outer layer, about 80nm thick, and a less dark core. Within the cell, this core is surrounded by electron-dense flocculent material (black arrows in Fig. 5B). Some microtubules can be seen around the base of the spine and some more at the periphery of the cell (white arrows in Fig. 5B). The cell nuclei are horseshoe shaped and the distal cytoplasm contains numerous small electron-dense membrane-bound granules. Underneath the spine the cell is attached to a thick basement membrane with numerous "hemidesmosomes" (Fig. 5A).

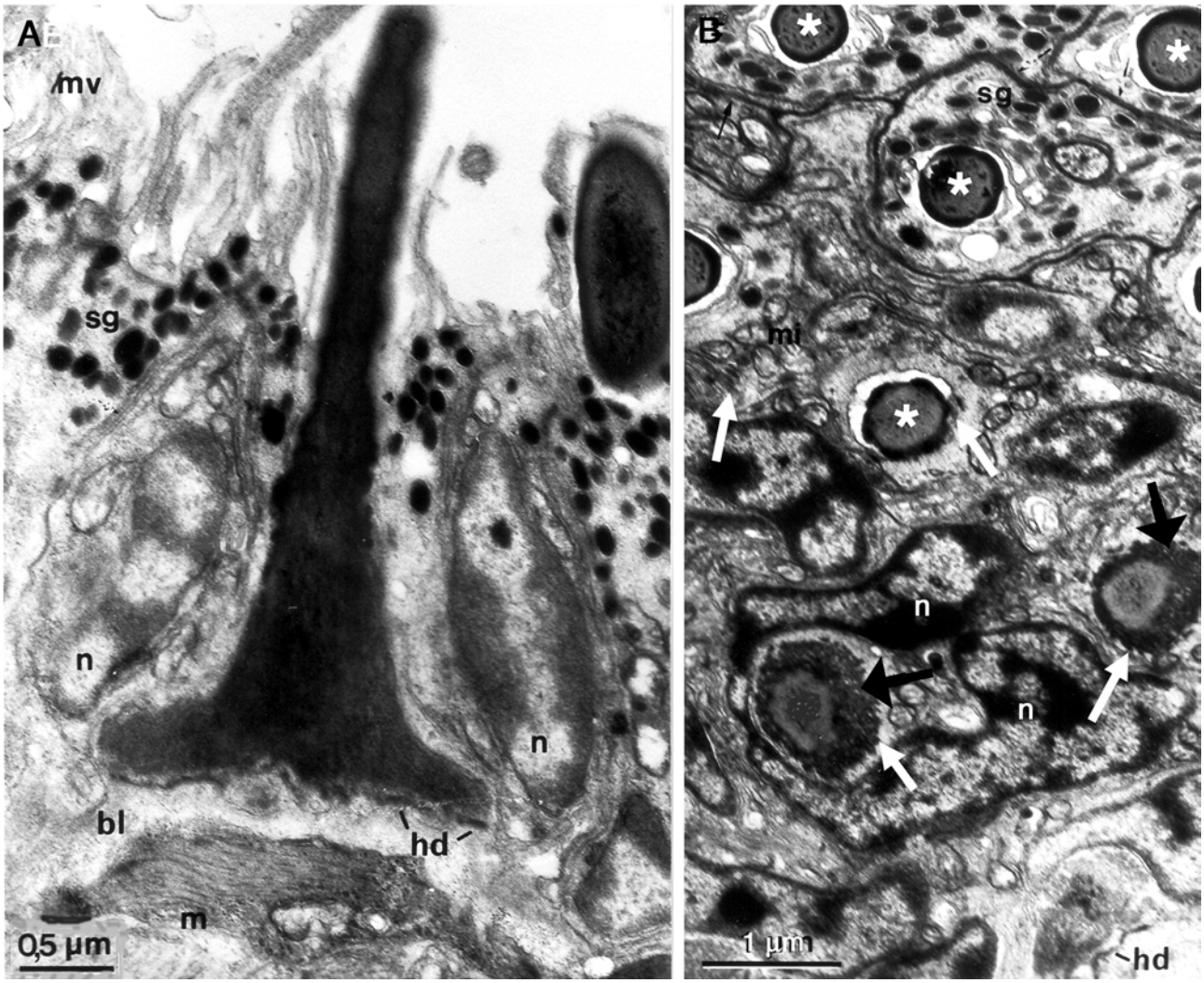

Fig. 5. – *Cirrifera genitoductus* n. sp.. Electronmicrographs of the cirrus spines. A: Longitudinal section through a spine in the cirrus. Note the "hemidesmosomes" with which the forming cell is attached to the thick basement membrane and the electron-dense granules at the apex of the cell. B: Cross sections at different levels of spines and forming cells in a tangential section through the cirrus wall. The black arrows point to the flocculent material at the base of the spines, the white arrows point to the microtubules and the * indicate the parts of the spines protruding from the forming cell. Note the horseshoe-shaped nucleus in A and in B.

The female system consists of a pair of globular ovaries in front of the pharynx and two rows of follicular vitellaria from just in front of the first testis follicle to about midway between pharynx and copulatory organ (Fig. 1). The two ovovitelloducts join just behind the copulatory organ to form a short common female duct, which receives the cement glands before it enters the atrium through its caudal wall (Fig. 3A). Just below the opening of the female duct is a short diverticulum of the atrium. From the junction of the oviducts a long genito-intestinal duct runs backwards and opens in the intestine behind the prostate vesicle and in front of the seminal vesicle (Fig. 3A,B).

The oviducts are two convoluting canals running next to the vasa deferentia. In the prepharyngeal region they are about 6µm wide, 10µm near their confluence. In any given cross section through an oviduct, its circumference is composed of only two cells, while that of the common duct is composed of four (and microvilli between the cilia), all with a very thin basement lamina and a weak muscle layer. Spermatozoa are frequently seen in the oviducts. The genito-intestinal duct is 9-12µm wide and has an epithelium similar to that of the common female duct, with four cells in cross-section, with microvilli, a very thin basement membrane and some weak muscles. All ducts in the female system have cilia.

DISCUSSION

The general anatomy of *C. genitoductus* n.sp. is like that of the majority of the representatives of the Coelogynoporidae: very long animals which may curl up, encapsulated brain, statocyst with four statocytes, numerous testes and vitellaria follicles, paired ovaries in front of the pharynx. The copulatory organ is clearly of the same construction as the members of the taxon *Cirrifera*: a long cirrus with many short spines, enclosed in a cirrus bulb and the seminal vesicle behind the copulatory organ. The ovovitelloducts enter a short common duct, which enters the atrium from behind. There is no bursa. Species of the taxa *Vannuccia* and *Stilivannuccia* also have a cirrus with many small spines enclosed in a bulb, but here the seminal vesicle(s) are in front of the cirrus. Moreover, there is a bursa in *Stilivannuccia*. *Macroatrium setosum* Riser, 1981 also has a cirrus and a posterior seminal vesicle, and it also has a bursa. The description of the latter species is, however, very elementary. *C. genitoductus* n.sp. deviates from all other *Cirrifera* species in that it has a long genito-intestinal duct. Main differences between the *Cirrifera* species can be derived from the identification key below:

Within several genera there are some species with a connection between the female system and the gut, while some other representatives of those genera lack such a connection. For example, in many species of *Coelogynopora* a bursa opens in the female duct, while in other species a duct running backwards from the common female duct opens in the gut: this is the so called genito-intestinal duct. This duct may be short or very long, ciliated or not and often a resorbing (bursal) tissue develops where the duct enters the intestine as in *Coelogynopora bresslaui* and *C. biarmata*, the two first coelogynoporid species described by Steinböck (1924). Such a genito-intestinal duct is also present in *Invenusta paracnida* Karling, 1966, in the species of *Ezona*, in *Vannuccia tripapillosa* and in *V. rotundouncinata* Ax & Sopott-Ehlers, 1979, while there is a bursa in *V. campana* Ehlers & Ehlers, 1980. In all previously-described species of *Cirrifera* and in the taxon *Carenscoilia* and in *Invenusta aestus* Sopott-Ehlers, 1976 the female system is very simple: both ovovitelloducts join each other behind the copulatory organ to form a short female duct, which opens in the atrium, and there are no bursal organs whatsoever. Now, in *Cirrifera genitoductus* sp.n., we have the first known example of a species of *Cirrifera* with a genito-intestinal duct.

1	– Seminal vesicles paired	2
	– Seminal vesicle unpaired	5
2.	– Prostate vesicle intracapsular (i.e. inside the copulatory bulb); spines of different sizes and shapes in the cirrus.	3
	– Prostate vesicle extracapsular, all spines have the same shape	4
3.	– Cirrus 120-130µm long, armed with spines 9-20µm long and with one large proximal spine, about 25µm long, with bifurcated base	*C. aculeata* (Ax, 1951)
	– Cirrus 80-100µm long, armed with three different types of spines: proximally about 20 curved spines each 4-9µm long, distally 60-70 spines, each 10-15µm long, and 2 or 3 lateral pairs of straight and axe-shaped spines in between, each 19-40µm long	*C. sopottehlersae* Noldt & Jouk, 1988
4.	– Cirrus 120-130µm long, spines of 7-12µm, lacking on the posterior wall in the distal part of the cirrus	*C. cirrifera* Sopott, 1972
	– Cirrus about 200µm long, uniformly armed with spines of 12-16µm	*C. xanthoderma* Riser, 1981
5.	– No prostate vesicle: prostate glands open in seminal duct close to copulatory bulb, but without a muscle coat. Cirrus mushroom-shaped, uniformly armed with spines of 8-13µm	*C. boletiformae* Sopott, 1972
	– An extra-capsular prostate vesicle between the seminal vesicle and the cirrus	
6	– Ejaculatory duct short, prostate vesicle close behind copulatory bulb. Cirrus 100µm long, spines 5-10µm but lacking spines on the anterior wall in the distal part of the cirrus	*C. dumosa* Sopott, 1972
	– Ejaculatory duct very long, prostate vesicle close to the seminal vesicle. Cirrus 130µm long, uniformly armed with spines 6-9µm long. With a genito-intestinal duct and a pair of large glands behind the genital pore	*C. genitoductus* sp. n.

Several other characters, of which we give some examples hereafter, also have such a "mosaic-like" distribution in the representatives of the Coelogynoporidae.

The copulatory organ may be a simple cirrus enclosed in a bulb with many small spines of similar size and shape, as in most species of the taxa *Cirrifera*, *Vannuccia* and *Stilivannuccia*. However, spines and needles of dissimilar size and shape occur in the copulatory organ of *C. sopottehlersae*. In most members of the taxon *Coelogynopora*, the cirrus (and/or atrium) bears long needles, either all similar or dissimilar, either attached to each other or not, while some species have a central stylet-like element surrounded by needles, the needles being free or attached to the stylet. The latter situation is also found in the taxon *Carenscoilia*. By contrast, a totally unarmed copulatory organ either cirrus or penis papilla occurs in some other species such as *Coelogynopora gynocotyla*, *Vannuccia tripapillosa* Tajika, 1977 and in the taxon *Invenusta*. In several species "accessory" stylets, either attached to a glandular reservoir or not, occur next to the copulatory organ proper, as in *Coelogynopora axi* Sopott, 1972, *C. hangoensis* Karling, 1953 and several other species of *Coelogynopora*, but also in the species of *Ezona* and of *Stilivannuccia*. The seminal vesicles may be paired or unpaired, and lie behind the copulatory organ in all coelogynoporids with the exception of in the species of *Vannuccia* and of *Stilivannuccia*. The vasa deferentia may fuse to a single seminal duct or remain separated until entering the seminal vesicle(s) from behind, in the middle or at its frontal side. The prostate glands may be within the copulatory bulb or enter the ejaculatory duct before it enters the copulatory bulb, and they may or may not be surrounded by a muscle sheath. A very long ejaculatory duct as in *Cirrifera genitoductus* (with extra-capsular prostate glands) is exceptional; it is also quite long in *Cirrifera aculeata*, but here the prostate vesicle is inside the copulatory bulb.

These examples of the variations within the Coelogynoporidae may suffice to demonstrate that making any statements about phylogenetic relations between the members of this taxon is premature. Including the taxa *Calviria* Martens & Curini-Galletti, 1993 and *Asilomaria* Karling, 1966 in the Coelogynoporidaae as proposed by CURRINI-GALLETTI (2001), based on molecular data, makes the picture even more confused. An attempt to perform a parsimony analysis, using the character states of about 30 characters, resulted in an unresolved bush (own data). Moreover, a sound comparison is difficult because many species have not been adequately described. An additional problem is that the degree of sexual maturity may cause differences that are easily overlooked or misinterpreted. Individuals of *Cirrifera genitoductus* sp.n. that we collected in March 1983 had only a short genito-intestinal duct, while it had grown to full length in the individuals collected later in the year. SOPOTT (1972) and AX & SOPOTT-EHLERS (1979) also mention that the bursal organs develop later than the male organs.

Our observations on the ultrastructure of *C. genitoductus* reveal a high degree of similarity with that of *C. aculeata*, in particular the ultrastructure of the cirrus spines. They consist of an intracellular floccular substance formed at the base of the cell, and its periphery is "smoothed" where it protrudes from the cell. Though we have no data on spine formation in sub-adults we can assume that the spines are formed in a "synchronous" way, indicated by the presence of microtubules and the forming cell that is attached to a thick basement membrane by halve-desmosomes immediately underneath the spine matrix (see BRUGGEMAN 1984; 1985 and in particular the discussions in his contributions of 1986 and 1988). Contrary to BRUGGEMAN's observations, the forming cell does not degenerate and remains active with the formation of the electron-dense granules found at the apical side of the cell (also in *C. aculeata*: fig. 12 of MARTENS & SCHOCKAERT, 1985). The significance of these granules remains unclear, but they are most probably not related to the formation of the spine.

In *C. aculeata*, two kinds of secretory granules have been observed in the glands of the prostate vesicle, as is the case in most flatworms. In *C. genitoductus* it looks as if the two glands are separated: one type in the prostate vesicle and the other type entering the ejaculatory duct near the copulatory bulb.

Basically, the ultrastructure of the epidermis is that of many free living Platyhelminthes (see RIEGER et. al., 1991 and the references therein). The feature of horizontal ciliary rootlets converging in a wedge of the anterior wall of the epidermis cells is now found in yet another proseriate species (own unpublished data); this remarkable situation might be an apomorphy for the Proseriata. The fine structures of the adhesive organs are similar to those of *Nematoplana coelogynoporoides* Meixner, 1938 (SOPOTT-EHLERS, 1979) and the receptors are like those of other Proseriata Lithophora (see SOPOTT-EHLERS, 1984). The large packages of glands at both sides behind the copulatory organ are, to the best of our knowledge, unique within the Proseriata. They consist of two kinds of glands, glands with large granules of low electron-density and glands with small electron-dense granules. They have an adhesive function (as observed in the living animal) and are reminiscent of the duo-gland adhesive glands (TYLER, 1976). Unfortunately, we have no data on the relations of the glands with the epidermis.

Many (all?) coelogynoporids have large glandular elements in the body wall that may look similar in the living animal, but are very different under the electron microscope. The so-called "paracnids" have been studied by SOPOTT-EHLERS (1981) in *Coelogynopora axi* and in *Carenscoilia bidentata* (1985). The ultrastructure of the "paracnids" in these two species is very different and SOPOTT-EHLERS (1985) claims to have unpublished data on the "paracnids" of the two *Invenusta* species, and that those are again very different. The large glands with hyaline content, seen in *Cirrifera genitoductus* may be confused with paracnids in the living animal. To the best of our knowledge such large hyaline glands do not occur in other coelogynoporid species, and paracnids do not occur in the taxon *Cirrifera*. These glands were described by SOPOTT in 1972 as "gelblichen (langlich) ovalen Hautdrusen", and were included in the genus diagnosis.

The diagnosis of the genus *Cirrifera* may be refined as follows:

Coelogynoporidae with intraepithelial nuclei and large hyaline glands in the epidermis. Male copulatory organ is a cirrus with many small spines, enclosed in a cirrus bulb. Seminal vesicle(s) caudal to the copulatory organ, paired

or unpaired. Prostate vesicle outside or within the cirrus bulb. Paired germovitelloducts join behind the copulatory organ and open in the common genital atrium through its caudal wall. There is no bursa but a genito-intestinal duct may be present.

Diagnosis of *Cirrifera genitoductus* n. sp.:

Cirrifera species with very long ejaculatory duct, prostate vesicle close to the seminal vesicle and additional prostate glands close to the copulatory bulb. Cirrus 130µm long, uniformly armed with spines 6-9µm long. With a long genito-intestinal duct and a pair of large glands behind the genital pore.

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ABBREVIATIONS USED IN THE FIGURES

a	genital atrium
ag	adhesive gland
bl	basement membrane
br	brain
cgp	common genital pore
ci	cirrus
cb	cirrus bulb
dgi	genito-intestinal duct
ej	ejaculatory duct
f	pharynx
fd	common female duct
fl	flagella of spermatozoon
g	hyaline glands
gu	gut lumen
hd	hemidesmosomes
lg	lateral gland complex
m	muscles
mi	mitochondrion
mv	microvilli
n	nucleus
o	ovary
od	oviduct
pd	pre-cerebral gut
pg	prostate glands
pv	prostate vesicle
rg	releasing gland
sd	seminal duct
sg	secretory granules
shg	"shell glands" or “cement glands”
st	statocyst
sv	seminal vesicle
sz	spermatozoon
t	testis
u	ultrarhabdite
vi	vitellaria
vd	vas deferens

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Construction of expression vector of human lactoferrin and its expression in bovine mammary epithelial cells

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ABSTRACT. Lactoferrin has multiple biological roles, including inflammation and immune modulation, iron absorption in newborns, non-iron-dependent antimicrobial activity, regulation of the level of retinoblastoma protein, protection against cancer development and metastasis, transactivation of the p53 tumor suppressor gene. Moreover, the ubiquitous expressed human lactoferrin (hLF) is species, tissue, and cell-type specific. To facilitate further studying of the biological function of hLF, we hereby constructed and expressed a recombinant vector containing exogenous hLF gene and bovine mammary gland ß-lactoglobulin (BLG) gene. cDNAs of both hLF gene (2259bp) and 5' flank regulatory fragment of BLG (1449bp) consisting of the promoter region, exon 1 and intron 1 of the gene were obtained respectively. Then the translation initiation codon of 5' flank regulatory fragment was mutated from ATG to AAG and renamed as mblg. hLF and mblg were recombined through PCR method and was named as BL, which was then cloned into pEGFP-C1 vector and renamed pMBL. Later on, the pMBL vector was transferred into a cell line of bovine mammary epithelia by liposomal transfection and cultured with G418 drug for 3 weeks to obtain positive transgene cell clones. Then the expression of hLF was detected in the positive cell clones by Immunocytochemistry and Western Blotting assays. The results showed that the exogenous hLF gene had been successfully integrated into the chromosome of the positive cell clones, which highly expressed hLF.

KEY WORDS : Bovine β-lactoglobulin (BLG) gene, point mutation, Human lactoferrin (hLF) gene, Recombinant PCR

INTRODUCTION

Lactoferrin gene is highly conserved among human, mouse, bovine and porcine species. Human lactoferrin (hLF) gene, at the human chromosomal 3p21 location (KLINTWORTH et al., 1998; YANG et al., 2003), is a non-haem iron-binding glycoprotein of 80kDa that belongs to transferrin family. Mature hLF protein contains 692 amino acids and its three-dimensional structure reveals a globular protein folded into two highly homologous parts (N-lobe and C-lobe), each of which can tightly bind a single ferric ion (Fe^{3+}) separately (ANDERSON et al., 1987). The genomic gene of hLF comprises 17 exons and spans about 35kb in genome (SEYFERT et al., 1994; KIM et al., 1998). It was first discovered in milk and later found in the wet surface mucosa epithelium (BAVEYE et al., 1999; VORLAND, 1999). Further studies showed that the expression of hLF is both ubiquitous and species, tissue, and cell-type specific (TENG, 2002). In the adult, hLF is mainly secreted by mammary gland and neutrophils. The highest levels of hLF are detected in colostrums and milk, with lower levels detected in tears, nasal fluids, saliva, pancreatic, gastrointestinal and reproductive tissue secretions (LEVAY & VILJOEN, 1995; TENG et al., 1989). Extensive in vitro and in vivo evidence has suggested that lactoferrin has multiple biological roles, including inflammation and immune modulation, iron absorption in newborns, non-iron- dependent antimicrobial activity, and regulation of the level of retinoblastoma protein (CONNEELY, 2001; BERLUTTI et al., 2006; ALYSHEV & VALYSHEVA, 2006; SON et al., 2006; WARD & CONNEELY, 2004; WARD et al., 2002). Moreover lactoferrin is also involved in protection against cancer development and metastasis, transactivation of the p53 tumor suppressor gene (TP53) and regulation of other gene expression (VORLAND, 1999; BEZAULT et al., 1994; ARTYM, 2006; SHIMAMURA et al., 2004; WANG et al., 2000). It can also promote bone growth and possibly modulates behavior in human and animals (NAOT et al., 2005; SACHARCZUK et al., 2005). hLF is a bioactive, versatile protein, and has large potential in nutritional and therapeutic applications, therefore the need for a recombinant source of hLF protein has increased and production of it using animal bioreactors has been studied widely to satisfy its large requirement (BAVEYE et al., 1999; VORLAND, 1999).

Transgenic animals provide an alternative approach to supply human lactoferrin in large quantity with relative low cost and animal mammary gland bioreactors are supposed to be the feasible tools for production of hLF in large scale. However, construction of an efficient and specific expression vector is the key link for the production of a mammary gland bioreactor. Several researchers have reported that hLF can be expressed in bovine, tobacco, rice plant, rat and other expression systems (KIM et al., 1998; ZAKHAROVA et al., 2005; TAKASE et al., 2005; CHOI et al., 2003; KUMURA et al., 1998; KRIMPENFORT, 1993) and transgenic animals can produce hLF in milk (KRIMPENFORT et al., 1991; PLATENBURG et al., 1994; KIM et al., 1999; PATRICK et al., 2002), but the expression level is not high enough to meet the needs. Therefore we

constructed a new specific expression vector containing the hLF gene and transfected it into bovine mammary epithelial cells, in this way, we have created a solid foundation for making transgenic animals.

MATERIALS AND METHODS

Bacterial strains and plasmids

Vector pMD 18-T and pEGFP-C1 were purchased from Clontech and TaKaRa respectively. *E. coli* DH5α, bovine mammary epithelial cells were preserved in our lab.

Primer sequences

Primers were designed based on the sequence of hLF (GenBank NOX53961; Rey et al., 1990) and bovine ß-lactoglobulin (BLG) (GenBank NOX14710; Jamieson et al, 1987) in GenBank using the software Prime Premier 5.0. The hLF sense primer, LFS1,5-CAGACCGCAGA-CATGAAACT -3 and antisense primer, LFA1, 5'-GCAATCCCCACCTTCAGCAG-3'. The BLG mutant primer is 5'-GC TGCAGCCAAGAAGTGCCT-3'. Ligation primers are as follows: BF2: -AGTTTCATGTCT-GCGGT CTGGGGAGGGACCTTGAGCTG-3' and BF3: 5'-CAGCTCAAGGTCCCTCCCCAGACCGCAGAC ATGAACT-3'. The detection sense primer, JCS1, 5'-CCT-CAGGGTGCCGAGTTGG-3' and anti-sense primer, JCA1, 5'-TTCAAGAATGGACGAAGTGT-3'.

Cloning and sequencing of human lactoferrin gene

The 2259bp full-length of human lactoferrin cDNA (GenBank accession No. AY165046) was cloned by reverse transcription polymerase chain reaction (RT-PCR) from human mammary carcinoma tissue obtained from Yangling Model District Hospital (Shannxi, China) after consent of the informed patients. Total RNA was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instructions and was reverse transcribed into cDNA with SuperScript™ II reverse transcriptase (Invitrogen) and oligo (dT) primer. Then the cDNA was amplified by PCR with primers LFS1 and LFA1. Thirty cycles of PCR were performed under the following conditions: denaturalization at 94 for 1min, annealing at 55 for 1min, and extension at 72 for 2min. The PCR product was verified by agarose gel electrophoresis and purified by gel extraction kit (Qiagen). Then the full-length hLF cDNA was ligated into pMD 18-T vectors and subjected to DNA sequence analysis.

Construction of bovine β-lactoglobulin (BLG) gene expression vector pBLG and point mutation of the 5' flanking fragment of BLG gene

Plasmid pBLG containing the 5' flanking fragment was constructed as previously described (Higuchi et al., 1988; Carl & Gabriela, 2003). Mutant site was introduced to BLG mutant primer when it was synthesized in vitro. The translation start codon of the 5' flanking fragment of bovine β-lactoglobulin (BLG) gene was mutated from ATG to AAG through point mutation with a primer-mediated mutagenesis procedure (Higuchi et al., 1988) The mutagenesis reaction was carried out following the method of references for creating point mutations (Carl & Gabriela, 2003). The mutant fragment was named mblg and cloned into vector pMD 18-T, and the mutant nucleotide acid in the pMBLG plasmid was confirmed by DNA sequencing and analyzing.

Construction and identification of hLF expression vector pMBL

The strategy of the construction of the mammary-specific expression vector of the recombinant hLF gene is shown (Fig. 1). First two ligation primers BF2 and BF3 were designed. The fragments mblg and hLF were ligated by recombinant PCR method (Carl & Gabriela, 2003). The PCR products were named BL with the ends containing the *Ase* I and *Hind* III restriction sites and ligated into the pMD 18-T vector as plasmid pBL. Later BL was excised and subcloned into the multiple cloning site of the pEGFP-C1 vector at *Ase* I and *Hind* III sites after the CMV promoter and GFP gene of vector pEGFP-C1 had previously been removed with restriction enzymes *Ase* I and *Hind* III. The recombined plasmid was designated as pMBL (Fig. 2), which is a mammary gland-specific expression vector. The pMBL was digested by *Mlu* I and the digested products were evaluated by agarose gel electrophoresis.

Cell culture and transfection

The plasmid pMBL was prepared by Endofree Plasmid Extraction Kit (Promega, USA) according to the manufacturer's instructions, and diluted in endotoxin-free PBS (Sigma). Bovine mammary epithelial cells were cultured in a 1:1 mixture of Ham's F12: Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), hydrocortisone (5μg/mL; Sigma), epidermal growth factor (10ng/mL; Sigma), insulin- transferrin- selenium sodium (5μg/mL; Sigma) and insulin-like growth factor (10ng/mL; Sigma) and maintained at 37 in 5% CO_2. For transfection procedure, cells at 50% confluence in 6-well plates were transfected with 4μg pMBL using the Lipofectamine 2000 reagent according to the manufacturer's instruction (Invitrogen). Cells were selected in growth medium containing 200μg/mL G418. After 3 weeks of selection, G418-resistant clones were selected randomly from the surviving colonies.

Detection of hLF gene stably integrated into chromosome of bovine mammary epithelial cells by PCR

To demonstrate whether hLF gene stably integrated into chromosome of bovine mammary epithelial cells, genomic DNA was extracted from the positive cloning cells according to the conventional method. The PCR amplification was performed using primers that amplified the specific 605bp fragment in pMBL plasmid DNA (sense primer JCS1 locates in gene BLG; antisense primer JCA1 is located in gene hLF).

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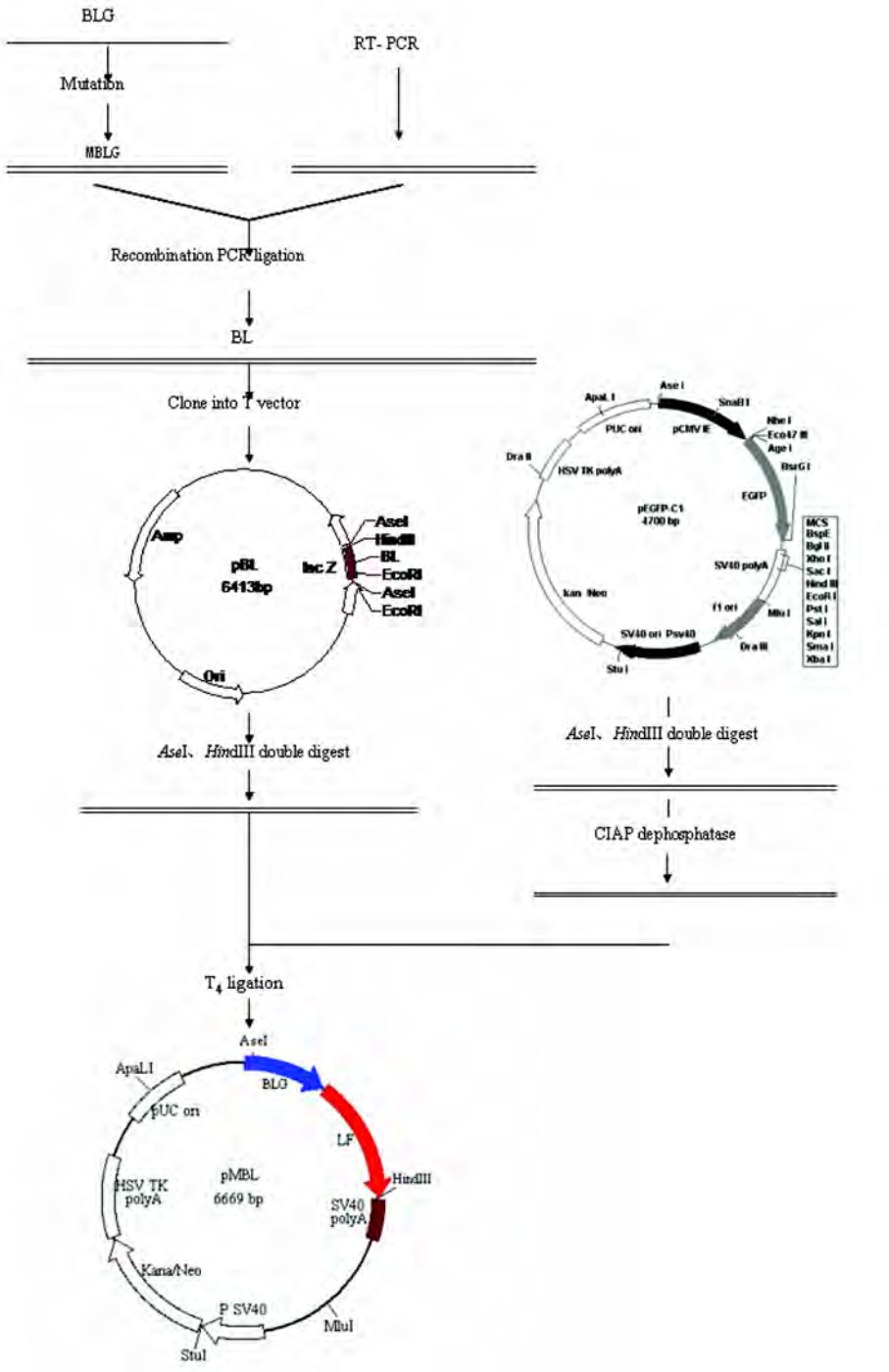

Fig. 1. – Strategy of the construction of the spedifically mammary expression vector of hLF gene.

Fig. 2. – Structure of human lactoferrin mammary specific expression vector pMBL
BLG P: bovine ß-lactoglobulin (BLG) promoter, E1: the exon of BLG gene, I1: the intron exon of BLG gene, hLF cDNA: cDNA of human lactoferrin gene, SV40 polyA: SV40 polyadenylation signal.

Detection of hLF stable expression by immunocytochemistry

The transfected cells were seeded on glass slides at 60-80% confluence, fixed in 4% paraformaldehyde for 10 minutes, blocked in sheep serum for 10 minutes, washed in 0.2% Triton X- 100 for 2 minutes and then immuno-stained with affinity-purified polyclonal rabbit antibody (Abcam) to human lactoferrin (1:200). For visualization, FITC-conjugated mouse anti-rabbit secondary antibodies (Sigma) were applied. Cellular nuclei were visualized by staining with 4,6-diamidino-2-phenylindole (Sigma) at 0.1μg/mL. Finally the cells were detected with a laser confocal microscopy (Leica).

Detection of hLF stable expression by western blotting

Western blotting was done according to the method of Kim (Kim et al., 1999). The cells were homogenized and the supernatant was separated by 15% PAGE (50μg total protein/lane). After transferred to nitrocellulose membranes, the membranes were blocked with 5% nonfat milk/PBS, followed by incubation at room temperature for 1h with polyclonal human lactoferrin antibody derived from rabbit (1:400) in 5% milk/PBS. After three 5min washes at room temperature, the membranes were incubated with HRP-conjugated secondary antibody (goat anti-rabbit IgG, 1:6000) in 5% milk/PBS. After three washes in PBS with tween-20, then followed by 5min of incubation with SuperSignal West Pico substrate (Pierce), the membranes were exposed on x-ray film and the signal was detected with Western Blotting Detection System (Bio-Rad), following the manufacturer's instructions. The expression of beta-actin protein was detected as normal expression control.

RESULTS

Construction of hLF expression vector pMBL

To construct the hLF expression vector pMBL, the translation initiation codon of the 5' flanking fragment of BLG gene was mutated from ATG to AAG (at positon 81) by PCR point mutation firstly. The mutant fragment was named mblg and cloned into pMD 18-T vector. The result was confirmed by DNA sequence analysis (Fig. 3). Secondly, the cDNA of hLF gene was cloned by RT-PCR. By agarose gel electrophoresis, it demonstrated that the cDNA was successfully amplified and the sizes of PCR products were correct (Fig. 4A). The PCR products were purified and cloned into pMD 18-T vectors to sequence and analyze the change of gene sequence. The sequence was aligned with the sequence registered in the GenBank based on Basic Local Alignment Search Tool (BLAST) analysis, and it showed that the sequence had 99.73% sequence identity and six point mutations: 100 (G to A), 155 (A to G), 458 (C to T), 1279 (T to G), 1752 (G to C) and 1909 (C to T). By Compared with human lactoferrin cDNA and its amino acids sequences, it showed the first five point mutations were allele mutation sites and the sixth point mutation was the change of synonyms codon. Thirdly, to demonstrate whether the BL fragment derived from mblg and hLF fragments connected together was inserted into the multi-clone sites of pEGFP-C1 vector correctly, the expression vector pMBL was digested by Mlu I and evaluated with agarose gel electrophoresis. The PCR product was a single approximate 6.6kb-fragment as showed in Fig. 4 B, it suggested that the BL fragment was successfully inserted into pEGFP-C1 vector.

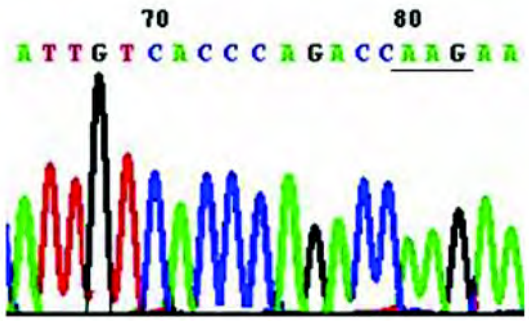

Fig. 3. – The sequencing chromatogram figure of the bovine BLG translation start code Note: The mutant nucleotide acids marked as underline.

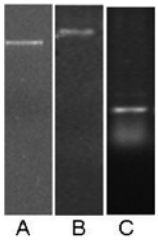

Fig. 4. – The figures of agarose gel electrophoresis.
Lane A: The PT-PCR product of hLF cDNA; Lane B: The expression vector pMBL digested with Mlu I; Lane C: Detection of exogenous hLF gene integrated into the genomes of the pMBL plasmid DNA transfected cells.

hLF gene stably integrated into chromosome of bovine mammary epithelial cells by PCR detection

To detect whether exogenous hLF gene was integrated into the genomes of the bovine mammary epithelial cells, the specific 605bp fragment was amplified by PCR. PCR analysis showed that specific fragment was amplified only from pMBL plasmid DNA transfected cells, but not from the cells without transfected (Fig. 4C).

hLF stably expressed in transfected cells by immunocytochemistry and western blot assays

Exogenous hLF gene expression in bovine mammary epithelial cells was detected by immunocytochemistry and western blot assays. After positive single clone cells were isolated and picked out by cultured with G418 drug for three weeks, the cells were coated with hLF antibody

and fluorescent antibody. Under confocal microscopes, it was observed that the fluorescence staining was positive in the cytoplasm of transfected cells and negative in the untransfected control cells (Fig. 5). Results of western blot also indicated that human lactoferrin, a 76kD protein, expressed highly in positive cells but negative in the controls (Fig. 6).

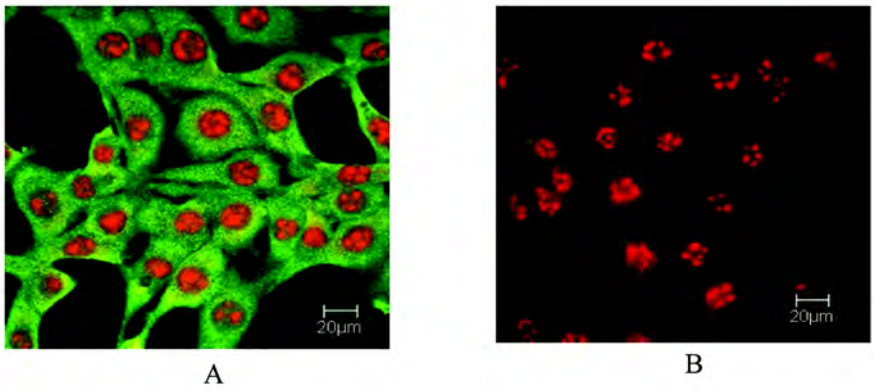

Fig. 5. – Detection of exogenous hLF gene expression in bovine mammary epithelial cells by immunocytochemistry. A: Transfection group and the fluorescence staining in the cytoplasm was positive, B. Negative control group of no transfection.

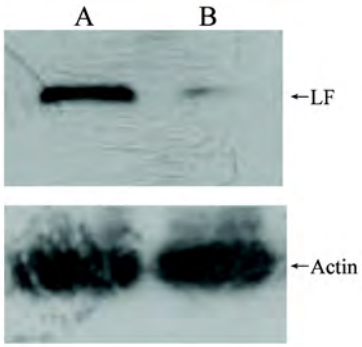

Fig. 6. – Detection of exogenous hLF gene expression in bovine mammary epithelial cells by western blot, the expression of actin detected as endogenous expression level. A: Transfection group, B: Negative control group of no transfection.

DISCUSSION

The key step of generation mammary gland bioreactor is to select appropriate regulatory elements. These regulatory elements enable high and specific expression of exogenous gene in the mammary gland of transgenic animal with subsequent secretion of the desired proteins in the milk. In this research, to evaluate the rationalization and expression efficiency of the expression vector, we transfected bovine epithelial cells cultured *in vitro* with the pMBL plasmid DNA and detected the expression level of exogenous hLF gene by immunocytochemistry and western blot assays. The results demonstrated that hLF gene had been integrated into the genome of transgenic cells and expressed at a very high level. As a result, it not only provides donor cell for somatic cell nuclear transfer to produce transgenic animal, but also takes the foundation of theory and practice for making transgenic animals and constructing efficient expression vector. We are attempting to prepare transgenic donor cell of hLF gene for producing transgenic calves by somatic cell nuclear transfer in the present study.

The 5' regulatory region of BLG gene is widely used to regulate exogenous gene expressed specifically in mammary epithelial cells (Van Kuik-Romeijn et al., 2000; Wright et al., 1991). In preparation for expression vector, because the regulatory sequence involves the first exon, expression of the recombinant gene would generate the BL fusion protein, resulting in decreased biological activity of lactoferrin. Therefore we mutated the translation initiation codon of the BLG gene from ATG to AAG. This mutation not only avoids the production of fusion protein but also mimics the natural transcription pattern of the bovine BLG gene through the first intron of the 5' flanking regulatory sequence. Several studies have confirmed that the first intron plays an important role in foreign gene transcription and correct splicing (Palmiter et al., 1982; Palmiter et al., 1992). In many studies the 5' regulatory sequence flanking the BLG gene of sheep or goat is used as the promoter of expression vector for producing transgenic animal. But few studies used the bovine 5' flanking regulatory sequence as promoter.

To construct an expression vector, we tried to connect the 5' flanking regulatory sequence of BLG with hLF gene using many methods. The conventional methods have many deficiencies when blunt ends are connected using T4 DNA ligase, such as low ligation efficiency and a high number of pseudopositive result**s.** This does not facilitate screening of positive clones. Using recombinant PCR to link two fragments is efficient and easy, but it requires the design of ligation primers and the optimization of the PCR reaction conditions. To avoid of mutation generated by PCR amplification, we used high faithful Platinum Taq DNA polymerase and reduced cycles of

PCR procedures (about 25 cycles). We selected unmutanted positive clones from lots of clones by sequencing and analysis. The result proved that the recombinant PCR was effective. In domestic reports, we have not found reports about using hLF as exogenous gene to construct mammary specific expression vector and transfect mammary epithelial cell cultured in vitro, also not found reports about transferring hLF gene in big transgenic domestic animals. Previous reports only used mouse, insect and plant to express hLF gene. In external reports, the reports about transferring hLF gene in big transgenic domestic animals are few.

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SHORT NOTES

Genetic variation within *Trogonophis wiegmanni* Kaup 1830

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The fossorial Amphisbaenid *Trogonophis wiegmanni* is the only extant representative of the family Trogonophidae in North Africa, occurring in the North of the Maghreb, from southwest Morocco to northeast Tunisia. Its elongate limbless body, reduced vision, compact skull and enhanced hearing and olfactory capabilities are morphological adaptations for burrowing (1). It is not commonly observed due to its fossorial habits and thus the distribution remains poorly known. Most authors accept the existence of two subspecies: *T. w. wiegmanni* and *T. w. elegans* (2;3). Morphologically, the two subspecies are very similar except for their colouration patterns. *Trogonophis w. wiegmanni* presents a ground yellow colour and *T. w. elegans* a whitish, grey or pink one (3). Nevertheless, the yellow pigmentation of *T. w. wiegmanni* disappears after a very short period of time in alcohol, so the two forms in preserved specimens cannot be separated morphologically (3). *Trogonophis w. wiegmanni* is found in central and eastern Morocco and into western Tunisia (3). The western range limit is thought to be the oriental mountains of the Rif and Medium Atlas (2). It is rarely found above 900m (3). This subspecies occupies relatively dry regions, with its western range limit apparently coinciding with the 600mm isohyet (3). *Trogonophis w. elegans* is endemic to Morocco, found in the north and west of the Atlas mountain chains up to an altitude of 1600m (2;3). This form lives in relatively moist regions influenced by the temperate Atlantic climate with an eastern limit apparently coinciding with the 700mm isohyet (3). These apparently very different ecological demands of both forms suggest a considerable step towards speciation (3). In fact, in a recent checklist, GANS (4) considers them as two different species: *T. wiegmanni* and *T. elegans*. There are two principal mountain chains in Morocco, the Rif mountains in the north of the country and further south in the country the Atlas, Medium Atlas, High Atlas and Anti-Atlas mountains that extend in a northeast/southwest direction. The two forms are considered to be separated by the Atlas mountains and there is no evidence of hybridization (2). The Atlas mountain chain was formed towards the end of the Miocene and at this time the Rif formed an island, separated from continental Africa (5). The Miocene Atlas uplift may provide a general explanation of differentiation and speciation in many northwest African species (6). Such a hypothesis of vicariance has been suggested to explain the observed differentiation in various species such as *Acanthodactylus erythurus* (7) and *Agama impalearis* (6). Many reptile species in this area, that have been studied phylogeographically, revealed unexpected high levels of mitochondrial DNA (mtDNA) sequence variation. In *Lacerta perspicillata* 12s rRNA uncorrected distances detected were between 5.2 and 6.6% (8) and in *Tarentola mauritanica* the 16s rRNA uncorrected genetic distances between subspecies reached 8%, and 5% between the north and south Morocco populations (9;10). In all studies the authors suggest that some of the genetic lineages identified were probably distinct species. In *Acanthodactylus erythrurus,* genetic distances between all populations range up to 3.1% within 12s rRNA, which was considered as substantial intraspecific variation (11). In *Agama impalearis*, the Agamid lizard, the 16s rRNA maximum uncorrected intraspecific divergence was 2.6% (6) which the authors considered that, combined with other factors, supported the recognition of two species. Thus there is a real need to assess other species in this region for cryptic genetic variation.

Phylogeographic studies of amphisbaenians are almost non-existent, however, in a recent study in another amphisbaenian, *Rhineura floridana,* very high genetic distances were observed between the two populations studied (9.27%) and also within one of the populations (7.34%) suggesting that the taxonomy should be reviewed (12).

The aim of this study was to assess the levels of genetic variation within *Trogonophis wiegmanni* using mtDNA sequence data, and determine if the proposed subspecies are monophyletic. We expect also to contribute towards the clarification of the recent alternative taxonomical hypotheses. By comparing this species with other recent phylogenetic studies of reptiles in North Africa, a biogeographic pattern of genetic variation across various species could be overlaid against predicted geological barriers to gene flow.

The number and geographic locations of the specimens used in this study are given in Table 1 and Fig. 1. Specimens collected in the field were identified to subspecies following BONS & GENIEZ (2). Digital photographs were taken, and then individuals were released after tail tips were collected. Total genomic DNA was extracted from tissue samples following the SAMBROOK et al. (13) protocol. Two mitochondrial gene regions were amplified, sequenced and analyzed: fragments of the 12s rRNA gene (387bp) and 16s rRNA gene (486bp). Polymerase Chain Reaction primers used in both amplification and sequencing were 12Sa and 12Sb and 16SL and 16SH from KOCHER et al. (14). Amplification conditions were the same as described by HARRIS et al. (15). Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus. Mitochondrial DNA sequences

were aligned by eye. Aligned sequences of the combined partial gene regions were 873 base pairs long. The data were imported into PAUP* 4.0b10 (16) for phylogenetic analysis. For the analysis of the combined data maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference were used. The approach outlined by HUELSENBECK & CRANDALL (17) was used to test 56 alternative models of evolution, employing PAUP* 4.0b10 and Modeltest (18). Once a model of evolution was chosen under the Akaike Information Criterion, following BUCKLEY & POSADA (19), it was used to estimate a tree using ML (20) with random sequence addition (100 replicates, TBR branch-swapping) and support for nodes estimated by bootstrapping with 100 replicates (21). A MP analysis was carried out (100 replicate heuristic search, TBR branch-swapping) with gaps treated as missing data and support for nodes estimated by bootstrapping with 100 replicates (21). The Bayesian analysis was implemented using MrBayes version 2.01 (22) which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) sampling approach. Bayesian analyses were conducted with random starting trees, run 0.5×10^6 generations, and sampled every 100 generations using the general-time reversible model of evolution with a model of among site rate variation. Two independent replicates were conducted and inspected for consistency to check for local optima (23).

Following BONS & GENIEZ (2) *T. w. wiegmanni* has a discontinuous distribution in Morocco; in the Debdou plateau north to the Mediterranean, and in the Middle Atlas mountains. Our new record of two specimens from the Moulouya river basin (Tr62, Tr72) largely fills the gap between these areas, indicating the distribution is probably continuous.

TABLE 1

Sample code and locality of specimens used for this study

Species	Locality	Code
Trogonophis wiegmanni elegans	Morocco- Oulad Brahim	Tr 2
Trogonophis wiegmanni elegans	Morocco - Oulad Brahim	Tr 3
Trogonophis wiegmanni elegans	Morocco - Oulad Brahim	Tr 4
Trogonophis wiegmanni elegans	Morocco- Asilah	Tr 5
Trogonophis wiegmanni elegans	Morocco- Moulay Idriss	Tr 6
Trogonophis wiegmanni wiegmanni	Morocco- Ain Beni Mathar	Tr 62
Trogonophis wiegmanni wiegmanni	Morocco- Tirnest	Tr 72
Trogonophis wiegmanni elegans	Morocco- Al jadida	Tr 1R
Trogonophis wiegmanni wiegmanni	Tunisia- El Kef	Tr 2R
Trogonophis wiegmanni wiegmanni	Morocco- Moulouya river mouth	Tr 139R
Trogonophis wiegmanni wiegmanni	Morocco- Berkane Oujda	Tr 761
Trogonophis wiegmanni wiegmanni	Morocco- Berkane Oujda	Tr768

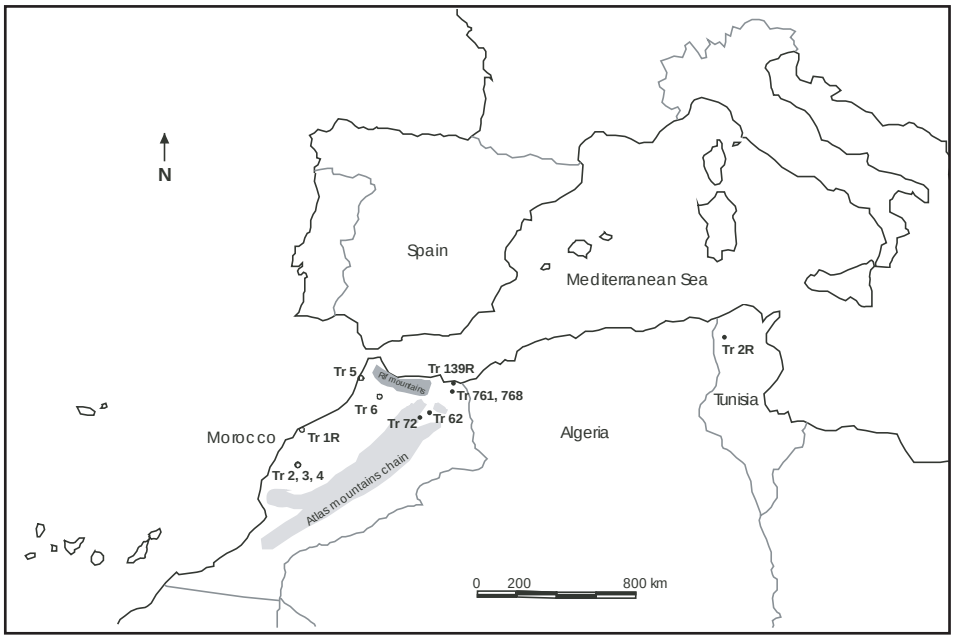

Fig. 1. – Map showing the sampling locations of *T. wiegmanni* sequenced for this study. *T. w. wiegmanni* is represented in full circles and *T. w. elegans* is represented in open circles. Codes are given in Table 1.

In total, 13 taxa were included for a total of 873 base pairs. Alignment was facile for both datasets, only 7 single base, 2 double and one 4 base pair insertions were needed. Sequences have been submitted to GenBank with accession numbers EF545712 to EF545735. *Trogonophis* is a monotypic genus; the closely related *Diplometopon zarudnyi* (24) was used to root the trees. We concluded that the General Time Reversible with a proportion of invariable sites and a discrete approximation of the γ distribution was the most appropriate model. A heuristic search incorporating this model inferred one tree of –ln 2146. Maximum parsimony estimated 3 trees of 198 steps the strict consensus of which was identical to the ML analysis, but less well resolved (Fig. 2). One hundred and three characters were parsimony informative. The estimate of phylogeny obtained using Bayesian analyses was identical to the ML tree. Our results provide evidence of two deep lineages corresponding to *T. w. elegans* and *T. w. wiegmanni* from Morocco (Bayesian probabilities and ML bootstraps for each: 0.97/86 and 0.94/64, respectively). The mean genetic distance between haplotypes in these two clades was high (3.8%). Evidence for the monophyly of these two clades was found in all the analyses.

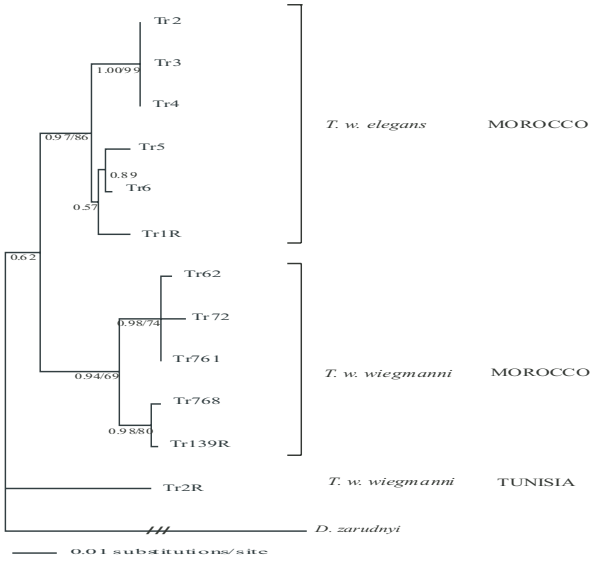

Fig. 2. – Tree derived from the ML analysis using the GTR+G+I model. All analyses produced identical relationships to the one shown. Below the branches, Bayesian posterior probabilities and bootstrap values for ML are indicated (posterior probabilities/ML bootstraps). For both analyses, only bootstrap values above 50% are represented.

The sample from Tunisia, thought to belong to *T. w. wiegmanni,* appears as a distinct lineage in all estimates of relationships. Concerning the genetic divergence, levels are high between the two Moroccan clades and the Tunisian individual (4.6% between the Tunisian individual and all the Morocco samples). The Tunisian individual is more divergent from *T. w. wiegmanni* (4.8%), the subspecies it is considered to belong to, than from *T. w. elegans* (4.4%).

Based on our analysis of 12s and 16s rRNA we confirm the existence of two monophyletic groups approximately separated by the Atlas mountains in Morocco. Compared to other species previously studied, the divergence between the two clades (3.8% for the combined data, 3.8% for 16s rRNA and 2.6% for 12s rRNA uncorrected distances) and between the Tunisian sample and the Moroccan groups (4.6% for the combined data, 4.8% for 16s rRNA and 4.3% for 12s rRNA uncorrected distances) can be considered high. Although not as high as the distances observed for *Tarentola mauritanica* (5% and 8% for 16srRNA uncorrected distances) and *Lacerta perspicillata* (12s rRNA uncorrected distances between 5.2 and 6.6%) that were considered cryptic species, divergences were higher than the ones reported for *Acanthodactylus erythrurus* (genetic distances between all populations range from 0.08 to 3.1% for 12S rRNA) and for *Agama impalearis* (16s rRNA maximum uncorrected intraspecific divergence of 2.6%). In the *Agama impalearis* phylogeographic study the sequence variation detected was considered surprisingly large and combined with other factors used to suggest the existence of distinct species (6).

In Morocco, this high genetic diversity combined with the well supported monophyly of the two mtDNA lineages and the apparently different ecological demands of both forms again raises the question of whether the two main clades could be considered distinct species. Furthermore, the morphological variation and the lack of morphological intermediate forms between subspecies (2) although weak, corroborates the two genetic lineages. Further sampling near the contact zone would be useful to confirm this. Field observations are also needed to better assess the ranges of the two forms, as our new records clearly indicate.

Concerning the Tunisian sample, our results do not conform to its inclusion in either of the Moroccan clades. The magnitude of mtDNA variation between the Tunisian sample and the other groups suggest that it belongs, at least, to a different subspecies possibly even to a different species. Unfortunately, the *T. w. wiegmanni* type locality has been described as restricted to Algeria (25). Since Algerian samples have not been included in this analysis, the correct nomenclature remains uncertain. Obviously, more sampling from Algeria and Tunisia will be crucial in evaluating the structure of the species *T. wiegmanni*, especially within *T. w. wiegmanni*. A detailed assessment of morphological variation is also needed, in particular between the Tunisian form and Moroccan *T. w. wiegmanni*. Only after this should the taxonomy be redefined.

These results are another example of high genetic variation within North African reptiles and indicate again the need for assessment of other Moroccan species in order to keep evaluating the diversity of this biogeographical complex region (9) and also to further address comparative biogeographic questions.

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A revised catalogue of the centipedes (Chilopoda) of the North Aegean Archipelago with particular reference to the islands of Híos, Límnos and Skyros

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Although there are numerous faunistic works concerning the centipede fauna of Greece (1, 2, 3, 4) and most of its islands (5, 6, 7, 8), there is still scanty information for many regions, such as the north Aegean archipelago. The centipedes of the north Aegean are almost unknown. Our poor knowledge derives mainly from scattered reports (1, 2, 3, 4, 5, 8, 9, 10).

The north Aegean archipelago consists of 6 large islands (>100km^2). Lésvos, the third largest Greek island and the seventh largest in the Mediterranean, is the largest and easternmost island of the area (1630km²), followed by Híos in the south with an area of 904km^2, Límnos, the most distant island from the continental areas with an area of 476km^2, Thásos, the northernmost island with an area of 380km^2, Skyros, the westernmost island of the region with an area of 209km^2, and Samothraki in the northeast of about 178km² (Fig. 1).

In the modern catalogue of the centipede fauna of Greece (4), one species has been recorded from Límnos, 2 from Skyros, 3 from Lésvos, 5 from Híos and Thásos as well as 7 from Samothraki.

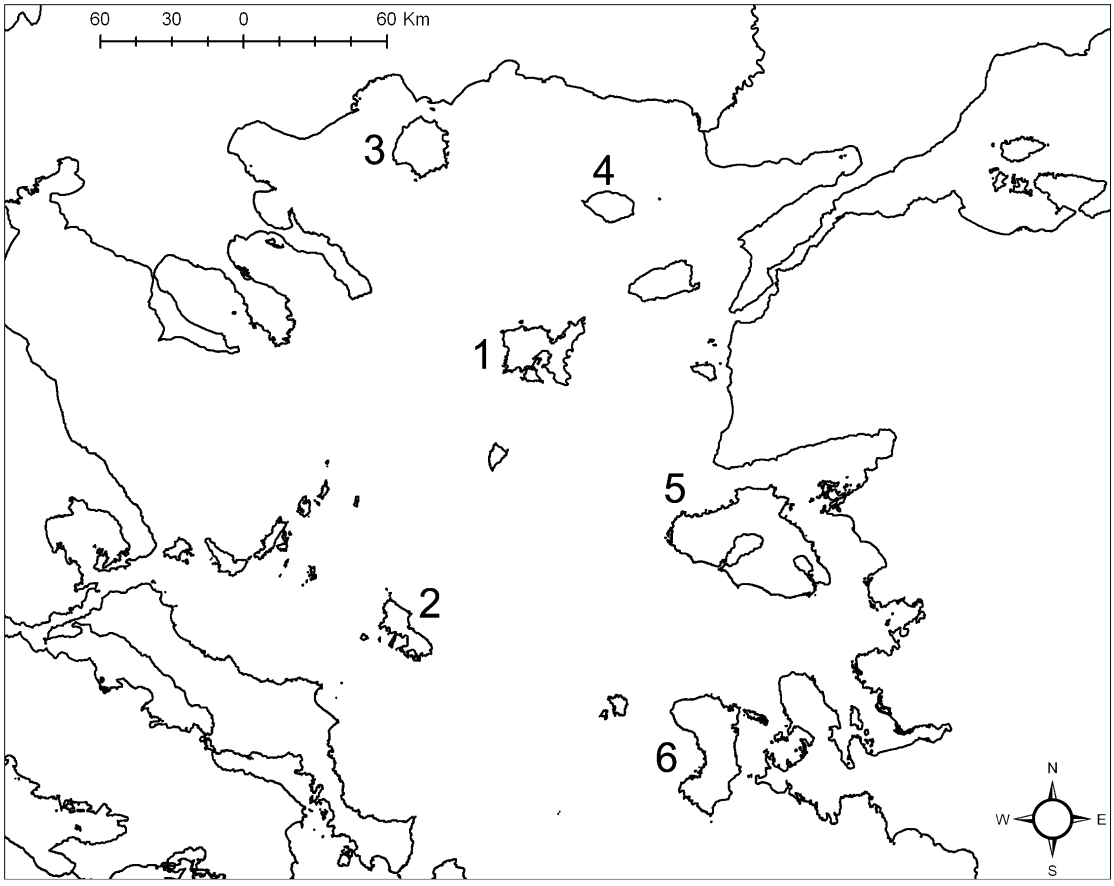

Fig. 1. – Map of north Aegean archipelago with the six largest islands numbered. 1: Límnos, 2: Skyros, 3: Thásos, 4: Samothraki, 5: Lésvos, 6: Híos.

Here, we present as complete a catalogue as possible of the centipede fauna of the north Aegean archipelago based mainly on unpublished records from Híos and Skyros deposited in the collection of the Natural History Museum of Crete (NHMC), and on recent records collected by the senior author on Límnos as well as on literature data.

TABLE 1

Presence of the centipedes in the six largest north Aegean islands, general distribution in the Aegean archipelago and the adjacent continental regions as well as chorotypes (6,7). NAeg: north Aegean, Dist: Distribution in the Aegean archipelago, M Gr: Mainland Greece, Turk: Turkey, BAL: Balkan, EN: Endemic, EUR: European, MED: Mediterranean, CEU: Central European, CSE: Central South European, CWP: Central West Palearctic, EME: East Mediterranean, NEM: North East Mediterranean, SEU: South European, SWA: South West Asiatic, SWP: South West Palearctic, WA: West Asiatic, N-S: north & south Aegean archipelago, N: distribution in the north Aegean archipelago, +: record based on literature data, f: first record for the island, n: new record for north Aegean archipelago, @: record based on recent and literature data, x: presence of species in mainland Greece or/and Turkey. Index numbers show the exact bibliographic reference.

	Híos	Lésvos	Límnos	Samothraki	Skyros	Thásos	NAeg	Chorotype	Chorotype	Dist	M Gr	Turk
Geophilomorpha								s.str	s.l.			
Dignathodontidae Cook, 1895												
1 *Henia athenarum* Pocock, 1891					f		n	NEM	MED	N-S	x	x
2 *Henia bicarinata* Meinert, 1870			f				n	CEU-MED	MED	N-S	x	x
3 *Henia illyrica* (Meinert, 1870)	f						n	SEU	EUR	N	x	x
Geophilidae Cook, 1895												
4 *Clinopodes flavidus* C. L. Koch, 1847	f	+(3)	f					CSE-WA	TUE	N-S	x	x
5 *Geophilus conjungens* Verhoeff, 1898			f		f		n	NEM	MED	N-S		x
6 *Geophilus naxius* Verhoeff, 1901					f		n	NEM	MED	N-S		x
7 *Pachymerium ferrugineum* C. L. Koch, 1835						f		n	CWP	WPA	N-S	x
8 *Tuoba poseidonis* (Verhoeff, 1901)			f					MED-SWA	MED	N-S		x
Himantariidae Cook, 1895												
9 *Bothriogaster signata* (Kessler, 1874)	@(13)			+(2)				EME-WA	TUM	N-S	x	x
10 *Himantarium gabrielis* (Linné, 1767)	@(13)			+(2)	@(1)			BAL-MED	MED	N-S	x	x
11 *Stigmatogaster gracilis* (Meinert, 1870)						+(8)		MED	MED	N-S		
12 *Thracophilus chiosensis* Stavropoulos & Matic, 1990	@(13)							EN	EN	N		
Lithobiomorpha												
Lithobiidae Newport, 1844												
13 *Eupolybothrus litoralis* (L. Koch, 1867)	f		@(8)		f	+(14)		BAL-EME	MED	N-S	x	x
14 *Eupolybothrus werneri* (Attems, 1902)					f		n	BAL	BAL	N	x	
15 *Lithobius agilis* C. L. Koch, 1847	f						n	EUR	EUR	N-S	x	x
16 *Lithobius aeruginosus* (L. Koch, 1862)			f				n	EUR-NEM	EUR	N-S	x	x
17 *Lithobius carinatus* L. Koch, 1862			f				n	EME	MED	N-S	x	x
18 *Lithobius erythrocephalus* C. L. Koch, 1847			f		f		n	EUR-WA	EUR	N-S	x	x
19 *Lithobius forficatus* (Linné, 1758)				+(5)				EUR	EUR	N-S	x	x
20 *Lithobius lucifugus* L. Koch, 1862	f				f		n	EUR-WA	EUR	N-S	x	x
21 *Lithobius microps* Meinert, 1868	f						n	EUR	EUR	N-S	x	x
22 *Lithobius nigripalpis* L. Koch, 1867	@(5)	+(3)						NEM	MED	N-S	x	x
23 *Lithobius tiasnatensis* Matic, 1973				+(13)				BAL	BAL	N		
24 *Pleurolithobius orientis* (Chamberlin, 1952)	f						n	NEM	MED	N-S	x	x
25 *Harpolithobius anodus* (Latzel, 1880)	f			+(5)		+(2)		SEU-NEM	MED	N-S	x	x
Scolopendromorpha												
Cryptopidae Kohlrausch, 1881												
26 *Cryptops anomalans* Newport, 1844	+(2)		f	+(8)				SWP	WPA	N-S	x	x
27 *Cryptops dianae* Stavropoulos & Matic, 1990						+(13)		EN	EN	N		
28 *Cryptops hortensis* Leach, 1815						+(2)		CWP	WPA	N-S	x	x
29 *Cryptops kosswigi* Chamberlin, 1952	f		f				n	EME	MED	N-S	x	x
30 *Cryptops trisulcatus* Brölemann, 1902	f		f		f		n	MED	MED	N-S		x
Scolopendridae Newport, 1894												
31 *Scolopendra cingulata* Latreille, 1829	f		f	+(4)	@(1)			MED-WA	TUM	N-S	x	x
Scutigeromorpha												
Scutigeridae Gervais, 1837												
32 *Scutigera coleoptrata* (Linné, 1758)	f	+(8)	f		f			SEU-MED	MED	N-S	x	x

More than 120 specimens of centipedes were collected from 30 different sites during field work in Límnos (from April to September 2006), approximately 100 specimens were collected from 20 different sites in Skyros (from January to May 2002), and 65 specimens were collected from 16 different sites in Híos (December 2006). The specimens were collected by hand and preserved in 95% alcohol. Identifications were carried out at the Natural History Museum of Crete (NHMC). The map of the area and the figures were drawn with Arc View GIS version 3.1 and Corel Draw 10.

We present a complete centipede list from the 6 largest islands of north Aegean archipelago (Lésvos, Híos, Límnos, Skyros, Thásos, Samothraki), the chorotype and the distribution for each species, new records for each island and the wider area of north Aegean archipelago as well as possible faunistic affinities with the adjacent continental regions (mainland Greece and Turkey) (Table 1). However, it was not the subject of this work to focus on the taxonomic status, the diagnosis, the general distribution and the ecology (habitat preferences) of certain species, mainly because all these data were presented in previous papers (3, 4, 6, 7, 8, 11, 12). The known composition of the centipede fauna of Híos, Límnos and Skyros, has dramatically increased the known diversity. Thus, the new data show 17 species from Híos, 13 species from Límnos and 12 from Skyros.

The number of previously undocumented species for the north Aegean region is relatively large. Thirty-two species are now known, belonging to 7 families and 16 genera. Sixteen species (50% of the centipedes) are recorded for the first time from the north Aegean archipelago. *Eupolybothrus werneri* and *Henia illyrica*, two widespread species in mainland Greece (4), are reported for the first time from insular Greece (collected on the island of Skyros and Híos, respectively). Twelve species are recorded for the first time from Híos and Límnos, respectively, and 10 from Skyros. Five species (*Henia illyrica, Thracophilus chiosensis, Eupolybothrus werneri, Lithobius tiasnatensis, Cryptops dianae*) are not distributed in the southern parts of Aegean, while the rest are widely distributed. Two of them are endemic (*Thracophilus chiosensis, Cryptops dianae*). The genus *Lithobius* is by far the most species-rich, being represented by 9 species on north Aegean islands (28% of the centipede species in the area) followed by *Cryptops* with 5 species (16% of the centipede species).

Chorotypes of the centipede species of the north Aegean archipelago are summarized in Fig. 2. Fifteen species (48%) examined from north Aegean Islands have Mediterranean affinities (s.l.), 7 species (22%) have European affinities (s. l.). Another 6% belong to the Balkan (2 species) element, 9% are west Palearctic (3 species), 6% Turano–Mediterranean (2 species) and 3% Turano–European (1 species). Endemic centipedes (6%) have been recorded from 2 islands, Híos (*Thracophilus chiosensis*) and Thásos (*Cryptops dianae*), respectively. As far as south Aegean islands are concerned almost the same pattern is presented. Thus, the dominant faunistic element is the Mediterranean s.l. accompanied by the European, the Balkan and the west Palearctic (7). Regarding the faunistic element of the adjacent continental areas, species with Mediterranean affinities (s.l.) predominate in Turkey (12), whereas species in the south Balkan Peninsula have mainly European (s.l.) characteristics (11).

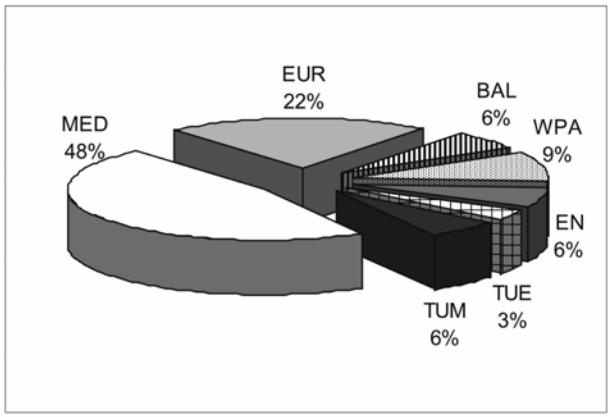

Fig. 2. – Chorotypes of the north Aegean centipede fauna (%). BAL: Balkan, EN: endemic, EUR: European, MED: Mediterranean, WPA: west Palearctic, TUE: Turano – European, TUM: Turano – Mediterranean.

Unlike the north Aegean centipedes (32 species), the centipede fauna of the southern Aegean archipelago (Crete, Kyklades, Dodekanisa) has been very well worked**:** 71 taxa from the south Aegean archipelago are already known (7). Therefore, it is apparent that the north Aegean region is still a field for future intensive faunistic research.

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Observations of the reduction of external gill filaments during larval development in *Heterotis niloticus*

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KEY WORDS : *Heterotis niloticus*, external gills, larval development, resorptions.

The arowana *Heterotis niloticus* (Cuvier, 1829) is distributed in the Sahelo-Sudanese freshwaters of West Africa (DAGET & DURAND, 1981; PAUGY, 2003) (1) (2). Until recently included within Osteoglossidae in the subfamily Heterotidinae, this species is now considered to belong to the Arapaimidae family, along with the south american pirarucú *Arapaima gigas* (Schinz) (FERRARIS, 2003) (3). Among other peculiarities, *H. niloticus* possesses externally projecting gill filaments at the earliest larval stages (DAGET, 1957) (4). These gills have an endodermal origin and seem to have a purely respiratory function. Although the presence of external gills has been reported in most major groups of aquatic anamniotes, they can be observed in teleosts in only one other species, *Gymnarchus niloticus* Cuvier (BUDGETT, 1901; ASSHETON, 1907) (5) (6). In Elasmobranchs, external filaments which float in the albuminous fluid are present during the embryonic phase, before the formation of the yolk sac. They appear to have a respiration as well as a food absorption function (BERTIN, 1958; PELPSTER & BEMIS, 1992) (7) (8). Smaller external gill filaments have been reported in Chondrosteans larvae (BERTIN, 1958; GISBERT, 1999) (7) (9). Analogous gills are present in juvenile Polypterids and in the larvae of the African and South American lungfishes (BERTIN, 1958; ROMER & PARSONS, 1986) (7) (10), but these external gills have an ectodermal origin and present a more complex structure which is very similar to those found in urodeles and caecilians. In anurans, two series of gills develop: the first consists of ectodermal external gills present at hatching. They regress and are replaced by endodermal internal gills during the tadpole stage. Finally, the branchial basket is completely resorbed during the metamorphosis (DEL CONTE & SIRLIN, 1952; PACKER, 1966; CHANNING, 1993) (11) (12) (13).

Samples of *Heterotis niloticus* were obtained from the Fishing and Aquaculture Institute of the University of Cheick Anta Diop (Dakar, Senegal). The fry was raised in natural conditions and collected in July 2005 and August 2005. Specimens were observed and dissected with a Leica M10 binocular microscope, and photographed with a Canon Powershot S50 camera.

At 6 hours post-hatching (PH), three branchial arches are present. Each bears several filaments of variable lengths. There is no trace of the opercular membrane and the arches are clearly visible in lateral view (Fig. 1). At 48 hours PH, gill filaments have lengthened and the opercular membrane is present. The fourth branchial arch is visible and bears some filaments. The longest filaments reach their maximal length at 72 hours PH. At this stage, the opercular membrane covers the two first branchial arches. At 96 hours PH, all the long filaments have dramatically regressed. The opercular membrane has increased in size but doesn't reach the fourth branchial arch. At 120 hours PH, all filaments have approximately the same size, and the opercular membrane completely covers the branchial basket.

The reduction of the gill filaments occurs simultaneously in all arches and appears to be progressive. There are two ways by which the obsolete tissues could be removed: it can either be lost, or it can be resorbed. In the case of a loss, the filaments would be broken at some point and the material would be freely released in the environment. In the case of a resorption, the size of the filaments would gradually decrease, as the material would be recovered to provide some energy used in the elaboration of new tissues. Here, it appears that the filaments are progressively decreasing in size after 72 hours PH until 120 hours PH, thus suggesting the occurrence of a phenomenon of resorption, with a probable recovery of the cellular material. Mechanisms of resorption that affect the whole body (involving hard and soft tissues) are already known in various teleosts during metamorphosis, such as in Carapidae (PARMENTIER et al., 2004) (14) and Elopomorpha (PFEILER, 1996, 1999; BISHOP & TORRES, 1999) (15) (16) (17). Material resorption affecting gill tissues was previously described in anuran tadpoles (WARKENTIN, 2000; BRUNELLI et al., 2004) (18) (19), but this work is the first to highlight a similar phenomenon in teleosts at larval stage.

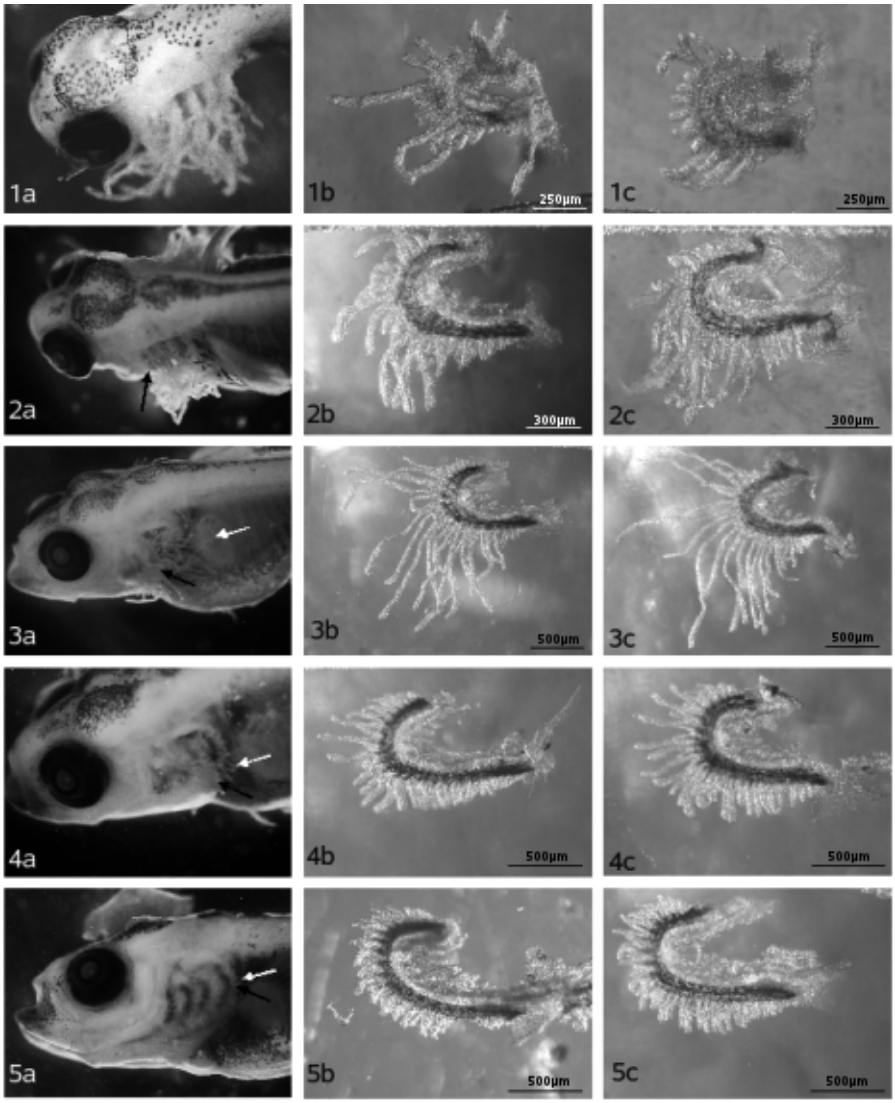

Fig. 1. – Photographs of *Heterotis niloticus* in external view (1a-5a) and of the first (1b-5b) and second (1c-5c) right branchial arch. **(1)** 6 hours PH. **(2)** 48 hours PH. **(3)** 72 hours PH. **(4)** 96 hours PH. **(5)** 120 hours PH. Black arrows show the distal tip of the opercular membrane. White arrows show the distal tip of the longest gill filament.

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