Contents

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SHORT NOTES

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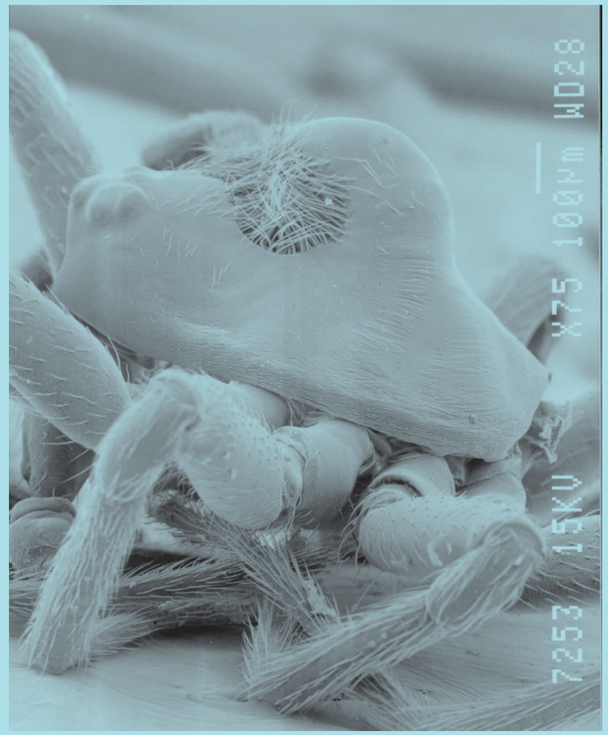

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Belgian Journal of Zoology

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Trophic habits and aquatic microhabitat use in gilled immature, paedomorphic and metamorphic Alpine newts (*Triturus alpestris apuanus*) in a pond in central Italy

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ABSTRACT. Current evolutionary models suggest that the presence of heterogeneous habitats favours the evolution of polymorphisms. In such cases, alternative phenotypes can coexist because they use different resources. Facultative paedomorphosis is a heterochronic polymorphism in which a morph – the paedomorph – retains larval traits during the adult stage while the other morph – the metamorph – is fully metamorphosed. The aim of this study was to determine the microhabitat use and the diet of Alpine newt paedomorphs, metamorphs and immatures (*Triturus alpestris apuanus)* coexisting in a small pond in Tuscany, central Italy, i.e. in a habitat where dimorphism is not expected. Although the two adult morphs do not use exactly the same resources, resource partitioning was weaker than in deep Alpine lakes. Nevertheless, the diet of immature gilled newts (larvae) differed from that of adults (metamorphs and paedomorphs). While the larvae eat a large number of planktonic organisms, the adults focus on insect larvae and newt eggs. The differences in resource use favour the coexistence of aquatic juveniles and adults. In the studied pond, facultative paedomorphosis was previously shown to be favoured by a precocious maturity of the paedomorphs. This study shows that the coexistence of paedomorphs and metamorphs may also be supported by some dietary and spatial segregation, although any advantages gained by this pattern are rather limited in the adult stage.

KEY WORDS : facultative paedomorphosis, resource partitioning, habitat, diet, size-selective predation, vacant niches, density regulation, newt.

INTRODUCTION

Newts live under water during the breeding period and adopt a terrestrial life during the rest of the year (Griffiths, 1996). Food and microhabitat use in the aquatic phase have been largely studied in the species of genus *Triturus* (see e.g. Griffiths & Mylotte, 1987; Joly & Giacoma, 1992). Newts often play the role of top predators (Schabetsberger & Jersabek, 1995). Their diet is wide, newts eating prey such as small crustaceans, molluscs, aquatic insect larvae, and aquatic insects (Joly, 1987a). Furthermore, they can also forage on amphibian eggs laid in the aquatic habitat (Joly, 1987a; Sattmann, 1989; Joly & Giacoma, 1992) and on terrestrial invertebrates that fall to the water surface (Chacornac & Joly, 1985; Sattmann, 1989; Joly & Giacoma, 1992; Schabetsberger & Jersabek, 1995). They can occupy all aquatic micro-habitats, i.e. the shoreline, the water column, the water surface and the bottom of lakes up to nine meters deep (Schabetsberger, 1993; Denoël & Joly 2001a).

Although they can be largely opportunistic, newts can also select microhabitats and prey according to specific taxa or sizes (Avery, 1968; Joly & Giacoma, 1992; Braz & Joly, 1994). In ponds where up to five newt species can be found (Arntzen & De Wijer, 1989), such habitat and prey selection might reduce species competition and then favour their coexistence (Schoener, 1974; Tokeshi, 1999). Resource partitioning has been observed in several newt communities composed of two (Dolmen & Koksvik, 1983; Griffiths & Mylotte, 1987; Dolmen, 1988) and three species (Joly & Giacoma, 1992; Fasola, 1993; Braz & Joly, 1994). However, large niche overlaps were also found in other newt communities (Braña et al., 1986; Griffiths, 1986, 1987; Jehle et al., 2000).

The life cycle of newts is complex and composed of a pre-metamorphic larval stage and a post-metamorphic juvenile and adult stage (Griffiths, 1996). However, in some populations, individuals forgo metamorphosis and reproduce in the larval stage (facultative paedomorphosis) (Semlitsch & Wilbur, 1989; Breuil, 1992; Whiteman, 1994). In Europe such a process is known in *Triturus* newts, including Italian populations of *T. alpestris* (Dubois & Breuil, 1983; Andreone & Dore, 1991; Andreone et al., 1993; Denoël et al. 2001b). Paedomorphosis has been shown to be favoured in permanent aquatic habitats at low densities (Harris, 1987; Semlitsch, 1987) and when prey are abundant (Denoël & Poncin, 2001). Early maturation of paedomorphs (Ryan & Semlitsch, 1998; Denoël & Joly, 2000), resource partitioning between morphs (Whiteman et al., 1996; Denoël et al., 1999; Denoël & Joly, 2001a), high energy intake (Denoël et al., 2002), different breeding

frequencies (Whiteman, 1997) and sexual activities in the two morphs (Denoël et al., 2001a) also favour the maintenance of facultative paedomorphosis in natural populations.

Resource polymorphisms are expected to be promoted in heterogeneous habitats devoid of competitors (Skulason & Smith, 1995; Smith & Skulason, 1996). This hypothesis is supported by empirical data in fishes (Malmquist, 1992; Malmquist et al., 1992; Robinson et al., 1993), and also in newts (Denoël et al., 1999, 2001b; Denoël & Joly, 2001a). Intramorphic differences in diet were also shown to favour coexistence of larval and paedomorphic newts in one of these lakes (Denoël & Joly, 2001b). However, in less complex habitats, space and feeding habits of paedomorphs and metamorphs have been poorly studied (e.g., Fasola & Canova, 1992) and no study has examined resource use in such sites occupied by only one species.

The aim of the present study was to determine the feeding and space habits of paedomorphic, metamorphic and immature gilled Alpine newts (*Triturus alpestris apuanus* [Bonaparte, 1839]) (Amphibia, Caudata, Salamandridae) coexisting in a habitat devoid of diversified components : a small pond. The comparisons of these traits in alternative heterochronic morphs will help us to understand the maintenance of facultative metamorphosis in such natural populations. Particularly, we expect large overlaps in resource use between adult morphs due to the small depth of the studied pond, but some partitioning between larval and adult stages because of size differences.

MATERIAL AND METHODS

The study site is located in the Apennines close to Parana (municipality of Mulazzo, Province of Massa Carrara, Tuscany, Italy; 44°17'N/9°51'E), at an elevation of 600 m a.s.l.. It is a small, shallow pond (maximum depth : 0.7 m, surface : 200 m^2; Fig. 1). Water level may decrease in summer, or, on some occasions, even dry up. The pond comprised two main microhabitats : an open area devoid of vegetation and an area covered with aquatic plants (*Glyceria*, *Typha*). The water surface freezes in winter, including at the beginning of the breeding season when there are still heavy snowfalls (March). The pond is surrounded by pastures and in the proximity of deciduous forests (around one hundred meters). It is devoid of fish and no water snakes were observed during the study. The newt community is only composed of Alpine newts *Triturus alpestris apuanus*, although we found a single individual of *Triturus carnifex carnifex* (Denoël et al., 2001b).

Five types of Alpine newt could be distinguished in the population : (1) (2) the male and female metamorphs, (3) (4) the male and female paedomorphs, and (5) the gilled larvae, also called gilled juveniles (overwintering individuals in the present analysis). Both paedomorphs and metamorphs were indeed sexually mature, as shown by behavioural analyses (Bovero et al., 1997; pers. obs.). Adulthood was here defined by the presence of a well developed cloaca (*vs.* a slit in juveniles). No metamorphosed juveniles were observed.

Fig. 1. – The study site (Parana, Tuscany, Italy; March 1997).

Newts were sampled with a landing net from an inflatable dinghy. Sampling effort was distributed according to a three-way design : (i) month, (ii) time of day, and (iii) microhabitat. Two microhabitats were sampled during each sampling session : the open area and the area covered by vegetation. Sampling lasted 20 minutes in each microhabitat and was carried out four times a day : at dawn (0600 hr), at mid-day (1200 hr), at the end of the day (1800 hr) and at midnight (0000 hr). Five sampling sessions were carried out in March 1997 (the fifth session at 1200 hr) and eight in April 1997 (two at each sampling time). These months correspond to the breeding period of the newts. At that time, both paedomorphs and metamorphs coexisted under water. Metamorphs are terrestrial outside the reproductive period. Captured newts were then stocked in four large containers filled with water from the pond. Immediately after the capture, a number of these animals (about eight individuals of each morph) were randomly drawn and anaesthetized in a solution of phenoxyethanol (0.5%). Stomach contents were then collected using a non-invasive stomach-flushing procedure (Joly, 1987b) and stocked in separate vials containing formaldehyde (4%). Prey were subsequently identified (at the species, genus, family or order level depending on taxa) and measured (total length) on squared paper under a stereoscopic microscope. Newts were measured (snout-vent length, SVL to the nearest mm) with a metal rule.

As data did not fit normal distributions, we used the Mann-Whitney *U*-test for all statistical comparisons of independent samples. Our samples being large and containing ties, we computed the normal approximation of the *U*-test. We also used Spearman rank correlation with associated *t*-test to determine the significance of relationships between variables. A chi-square test was performed to test for equal distribution of newts in the two microhabitats (Siegel & Castellan, 1988; Statsoft France, 2000).

Prey niche overlap between morphs was calculated using Schoener's (1970) index, which has already been used in newt ecology studies (Griffiths & Mylotte, 1987; Fasola, 1993) :

$$C = 1 - 0.5\left(\sum_i \left|p_{xi} - p_{yi}\right|\right)$$

where p_{xi} is the proportional utilization of prey type i by morph x, and p_{yi} the proportional utilization of prey type i by morph y. The index ranges from 0 (no prey in common) to 1 (all prey in common).

RESULTS

The four different forms of adult newt ($N = 358$) inhabited both the open and vegetation areas. Nevertheless, they significantly differed in the use of these microhabitats ($\chi^2 = 18.3$, 3 d.f., $p < 0.001$; Fig. 2). The paedomorphs were proportionally more abundant in the vegetation area ($\chi^2 = 12.82$, 1 d.f., $p < 0.001$; Fig. 2).

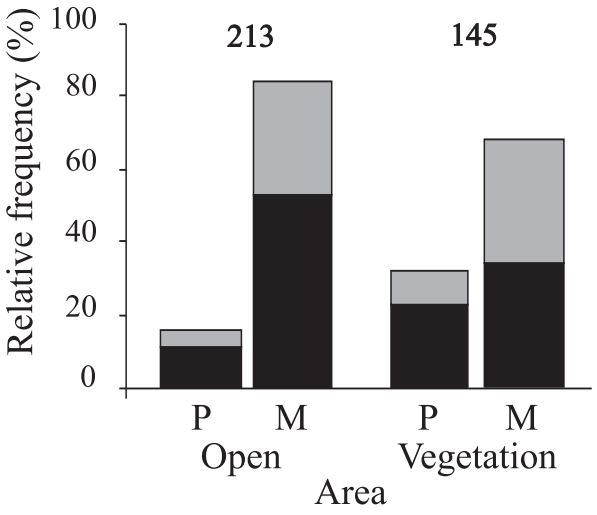

Fig. 2. – Spatial use of the two main microhabitats (open and vegetation areas) in the Parana pond in April 1997. P : paedomorph, M : metamorph; black bars : females, open bars : males.

The distribution of newts significantly differed at midday ($\chi^2 = 10.59$, 3 d.f., $p < 0.05$; Fig. 3) and at the end of the day ($\chi^2 = 11.51$, 3 d.f., $p < 0.01$; Fig. 3), but not at midnight ($\chi^2 = 3.68$, 3 d.f., NS; Fig. 3) or at dawn ($\chi^2 = 2.09$, 3 d.f., NS; Fig. 3). At midday and in the evening, paedomorphs were proportionally more abundant in the vegetation (respectively, $\chi^2 = 7.39$, 1 d.f., $p < 0.01$ and $\chi^2 = 10.14$, 1 d.f., $p < 0.01$).

A total of 7827 prey were obtained from the 296 newts (254 adults and 42 juveniles). The prey consisted mainly of crustaceans (Chydoridae, Cyclopoida, Ostracoda), insect larvae (chironomid and ceratopogonid Diptera, Plecoptera, Zygoptera, dytiscid and helodid Coleoptera), Alpine newt eggs, and sloughs of common toad *Bufo bufo* and newt *Triturus alpestris* (Fig. 4).

From a qualitative point of view, the two adult morphs foraged on the same kinds of prey (Table 1 and 2). Food niche overlap was also quite large : 0.75 between paedomorphic and metamorphic females and 0.74 between paedomorphic and metamorphic males. From a quantitative point of view, there were almost no significant differences between the two adult morphs (*U*-test, Table 1 and 2). Paedomorphic and metamorphic females significantly differed in prey use for chironomid, helodid and Zygoptera larvae, but not for the other prey (*U*-test, Table 1).

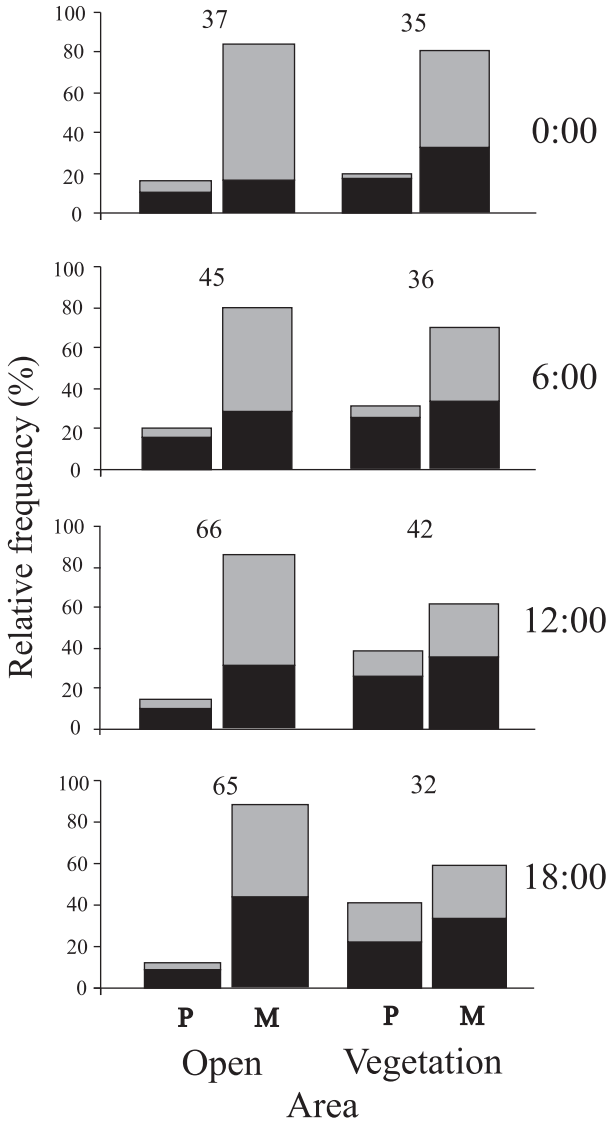

Fig. 3. – Temporal use of the two main microhabitats (open and vegetation areas) in the Parana pond in April 1997 : at midnight, dawn, mid-day and in the evening. P : paedomorph, M : metamorph; black bars : females, open bars : males.

The large mean number of *Chydorus* in paedomorphic females was due to only one individual that ate 259 specimens of this taxon. Paedomorphic and metamorphic males significantly differed in prey use of Chydoridae, Cyclopoida and amphibian sloughs, but not for the other prey (*U*-test, Table 2). In all significant cases, the scores were higher in the paedomorphs than in the metamorphs.

Significant differences were also observed between the two different sampling dates. In females, they concern only chironomid larvae. Other significant differences were found only in March for chironomid pupae and Zygoptera larvae, and in April for helodid and dytiscid larvae (Table 1). In males, no significant differences were found over the two months. In March, the two morphs differed in terms of amphibian sloughs and in April for chydorids, Cyclopoida and helodid larvae (Table 2). In females of the two morphs, food niche overlap was 0.66 in March and 0.68 in April. In males, it was 0.67 in March and 0.61 in April.

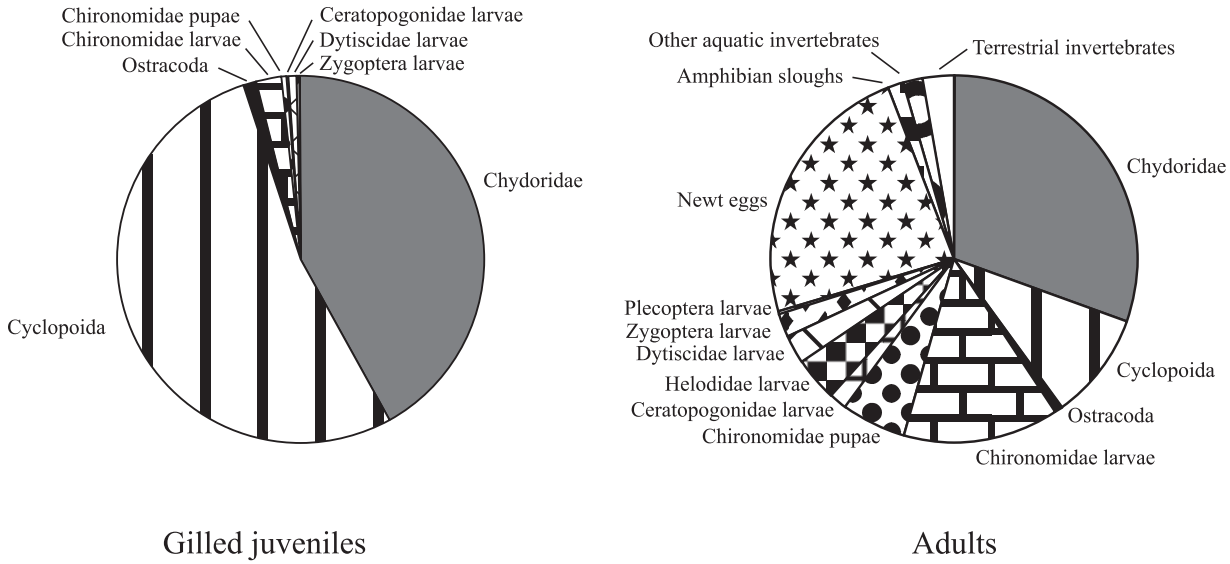

Fig. 4. – Relative composition of the gut contents of gilled juveniles ($N = 42$) and adults (paedomorphs and metamorphs; $N = 254$) in the Parana pond (March/April 1997). Prey accounting for less than 0.1 percent of the diet are not represented (i.e. Helodidae larvae, other aquatic and terrestrial invertebrates in branchiate juveniles). The large proportion of *Chydorus* in adults is due to only one individual.

Paedomorphic females consumed significantly more chironomid larvae ($p < 0.05$) and dytiscid larvae ($p < 0.01$) in the open area and more helodid larvae in the vegetation area ($p < 0.05$) than metamorphic females did (*U*-test). Paedomorphic males consumed significantly more Cyclopoida and helodid larvae in the open area ($p < 0.01$) and more *Chydorus* ($p < 0.01$) in the vegetation area than metamorphic males did (*U*-test).

There was no significant difference in diet between the two morphs, in males and females, in the midnight sample (*U*-test). Significant differences were found in the three other samples, except for males at dawn. Paedomorphic females ate significantly more chironomid larvae at dawn (*U*-test; $p < 0.01$) and midday; more helodid larvae at dawn and in the evening; and more dytiscid larvae at dawn and midday than the metamorphic females (*U*-test; $p < 0.05$). Stomachs of paedomorphic males contained more *Chydorus* ($p < 0.05$) and chironomid larvae ($p < 0.01$) at midday, and more amphibian sloughs in the evening than those of metamorphic males (*U*-test; $p < 0.05$).

Gilled juveniles ingested significantly more *Chydorus*, Cyclopoida, Ostracoda, and chironomid and dytiscid larvae than the adults (paedomorphs and metamorphs taken together), but they consumed fewer newt eggs (*U*-test, $p < 0.001$ for each of the six comparisons; Fig. 4). Food niche overlap was only 0.25 between adults and gilled juveniles.

There was a significant correlation between the size of the prey and the size of the newts (all individuals considered : $r_S = 0.32$, $t_{276} = 5.620$, $p < 0.001$). Mean size of the prey caught by paedomorphic and metamorphic males (5.9 mm and 7.5 mm respectively, *U*-test, $p = 0.05$) and by paedomorphic and metamorphic females did not differ significantly (6.9 mm and 8.6 mm respectively, *U*-test, $p = 0.5$), but differed between branchiate juveniles and adults (mean = 2.7 mm and 7.3 mm respectively, *U*-test, $p < 0.001$).

DISCUSSION

From our study it is evident that some differences were found in both spatial and food use between paedomorphic and metamorphic Alpine newts. Paedomorphs preferred the microhabitats with aquatic vegetation, while metamorphs preferred the open area devoid of vegetation. Some prey taxa were used differentially by the two morphs but not consistently across the sexes (chironomid, helodid, dytiscid and Zygoptera larvae in females and chydorids, Cyclopoida and amphibian sloughs in males). However, resource partitioning is different to interpret, since it is rather limited and does not appear in all subsamples. The overlap for food use between the adult morphs ranged between 0.61 and 0.75 (Schoener's Index, 1970). The two morphs were largely present in the two microhabitats and qualitatively used the same prey (i.e. mainly insect larvae and newt eggs, plankton being under-exploited). In fact, the difference in prey use was due to a larger capture rate by paedomorphs. Indeed, all significant comparisons between the two morphs revealed a higher mean prey number per stomach in paedomorphs than in metamorphs.

These results differ from those obtained in a deep Alpine lake (La Cabane lake, France). In such a diversified aquatic habitat containing a vertical component, paedomorphs foraged in all the micro-habitats, but metamorphs were limited to peripheral areas. The diet of paedomorphs was mainly composed of planktonic organisms (e.g., daphnids, Cyclopoida, chirocephalids). Metamorphs relied for a large part on terrestrial invertebrates that fell to the water surface of the lake (Denoël et al., 1999; Denoël & Joly, 2001a). Although less pronounced, a similar degree of diet and habitat segregation was also found in two other Alpine lakes in Greece (Denoël, 2001). As in our study, Fasola & Canova (1992) found a large overlap in food resources in the adult morphs occupying small ponds inhabited by three species

TABLE 1

Stomach contents of female paedomorphs (N = 35 in March, N=40 in April) and metamorphs (N = 29 in March, N = 39 in April) in a small Italian pond (Z-adjusted Mann-Whitney U-test)

Prey taxa	Month	Paedomorphs Mean (min – max)	Metamorphs Mean (min – max)	Z-adj.	p
Chydoridae	March	0	0.1 (0-2)	-1.100	0.27
	April	7.2 (0-259[a])	0.2 (0-4)	1.534	0.12
	Total	3.8 (0-259[a])	0.1 (0-4)	0.980	0.32
Cyclopoida	March	0.4 (0-6)	0.5 (0-5)	-0.939	0.34
	April	1.2 (0-16)	0.3 (0-4)	1.643	0.10
	Total	0.8 (0-16)	0.4 (0-5)	0.652	0.51
Ostracoda	March	0.1 (0-1)	0.1 (0-1)	0.25	0.81
	April	0.03 (0-1)	0.1 (0-1)	-0.607	0.54
	Total	0.1 (0-1)	0.1 (0-1)	-0.142	0.89
Chiromidae larvae	March	1.4 (0-6)	0.4 (0-3)	2.860	<0.01
	April	1.2 (0-7)	0.5 (0-5)	2.004	<0.05
	Total	1.4 (0-7)	0.5 (0-5)	3.437	<0.001
Chironomidae pupae	March	1.4 (0-11)	0.4 (0-5)	2.040	<0.05
	April	0.4 (0-3)	0.6 (0-4)	-1.031	0.30
	Total	0.8 (0-11)	0.5 (0-5)	0.767	0.44
Ceratopogonidae larvae	March	0.1 (0-2)	0.1 (0-2)	-0.200	0.84
	April	0.1 (0-1)	0.1 (0-1)	0.430	0.67
	Total	0.1 (0-2)	0.1 (0-1)	0.166	0.87
Helodidae larvae	March	0.9 (0-15)	0.4 (0-6)	1.345	0.18
	April	0.8 (0-14)	0	3.313	<0.001
	Total	0.8 (0-15)	0.2 (0-6)	3.265	<0.01
Dytiscidae larvae	March	0.2 (0-2)	0.1 (0-1)	0.910	0.13
	April	0.3 (0-3)	0.03 (0-1)	2.873	<0.01
	Total	0.3 (0-3)	0.04 (0-1)	3.158	<0.01
Zygoptera larvae	March	0.3 (0-4)	0	2.530	<0.05
	April	0.3 (0-2)	0.1 (0-1)	1.487	0.13
	Total	0.3 (0-4)	0.1 (0-1)	2.675	<0.01
Plecoptera larvae	March	0.2 (0-2)	0.2 (0-2)	0.670	0.50
	April	0	0.1 (0-2)	-1.012	0.31
	Total	0.1 (0-2)	0.1 (0-2)	0.430	0.67
Alpine newt eggs	March	1.0 (0-9)	1.3 (0-15)	0.027	0.98
	April	2.2 (0-13)	2.4 (0-17)	0.555	0.58
	Total	1.6 (0-13)	1.9 (0-17)	0.388	0.70
Amphibian sloughs	March	0.3 (0-1)	0.3 (0-1)	-0.758	0.45
	April	0.1 (0-1)	0.1 (0-1)	-0.032	0.97
	Total	0.2 (0-1)	0.2 (0-1)	-0.488	0.625
Other aquatic invertebrates	March	0.4 (0-3)	0.3 (0-5)	0.923	0.36
	April	0.2 (0-2)	0.2 (0-2)	0.258	0.80
	Total	0.2 (0-3)	0.2 (0-5)	0.904	0.37
Terrestrial invertebrates	March	0.1 (0-2)	0.2 (0-1)	-0.604	0.55
	April	0.3 (0-2)	0.2 (0-2)	0.665	0.51
	Total	0.2 (0-2)	0.2 (0-2)	0.145	0.89

TABLE 2

Stomach contents of male paedomorphs (N = 17 in March, N=27 in April) and metamorphs (N = 30 in March, N = 37 in April) in a small Italian pond (Z-adjusted Mann-Whitney U-test)

Prey taxa	Month	Paedomorphs Mean (min – max)	Metamorphs Mean (min – max)	Z-adj.	p
Chydoridae	March	0	0	-	-
	April	0.6 (0-4)	0.1 (0-1)	2.692	<0.01
	Total	0.4 (0-4)	0.03 (0-1)	2.768	<0.01
Cyclopoida	March	1.4 (0-12)	0.4 (0-4)	0.135	0.89
	April	1.4 (0-13)	0.1 (0-2)	2.589	<0.01
	Total	1.4 (0-13)	0.3 (0-4)	1.969	<0.05
Ostracoda	March	0	0.1 (0-1)	-1.076	0.28
	April	0.1 (0-1)	0.03 (0-1)	1.362	0.17
	Total	0.1 (0-1)	0.05 (0-1)	0.531	0.59
Chironomidae larvae	March	1.1 (0-4)	0.9 (0-3)	0.366	0.71
	April	1.1 (0-5)	1.1 (0-6)	0.111	0.91
	Total	1.1 (0-5)	1.0 (0-6)	0.289	0.77
Chironomidae pupae	March	0.8 (0-4)	0.3 (0-2)	0.812	0.42
	April	0.2 (0-3)	0.4 (0-4)	-0.941	0.35
	Total	0.4 (0-4)	0.3 (0-4)	-0.211	0.83
Ceratopogonidae larvae	March	0.2 (0-2)	0.2 (0-2)	-0.393	0.69
	April	0.2 (0-3)	0.1 (0-1)	0.203	0.84
	Total	0.2 (0-3)	0.2 (0-2)	-0.114	0.91
Helodidae larvae	March	0.3 (0-2)	0.4 (0-5)	0.320	0.75
	April	0.3 (0-1)	0.03 (0-1)	2.753	<0.01
	Total	0.3 (0-2)	0.2 (0-5)	1.0909	0.06
Dytiscidae larvae	March	0.2 (0-1)	0.5 (0-3)	-1.428	0.15
	April	0.3 (0-2)	0.1 (0-1)	1.315	0.19
	Total	0.3 (0-2)	0.3 (0-3)	-0.206	0.84
Zygoptera larvae	March	0.1 (0-1)	0	1.328	0.18
	April	0.1 (0-1)	0.1 (0-1)	0.038	0.97
	Total	0.1 (0-1)	0.1 (0-1)	0.619	0.54
Plecoptera larvae	March	0	0.03 (0-1)	-0.753	0.45
	April	0.03 (0-1)	0	1.171	0.24
	Total	0.02 (0-1)	0.02 (0-1)	0.301	0.76
Alpine newt eggs	March	0.1 (0-1)	0.1 (0-1)	-0.503	0.61
	April	0.9 (0-4)	1.0 (0-16)	0.860	0.39
	Total	0.6 (0-4)	0.6 (0-16)	0.785	0.43
Amphibian sloughs	March	0.6 (0-2)	0.2 (0-1)	2.374	<0.05
	April	0.2 (0-1)	0.1 (0-1)	1.234	0.22
	Total	0.3 (0-2)	0.1 (0-1)	2.363	<0.05
Other aquatic invertebrates	March	0.1 (0-1)	0.4 (0-4)	-0.590	0.55
	April	0.2 (0-1)	0.03 (0-1)	1.769	0.08
	Total	0.1 (0-1)	0.2 (0-4)	0.638	0.52
Terrestrial invertebrates	March	0	0.1 (0-1)	-1.076	0.28
	April	0.2 (0-1)	0.1 (0-1)	0.147	0.83
	Total	0.1 (0-1)	0.1 (0-1)	-0.233	0.82

Chydoridae are Cladocera, Cyclopoida are Copepoda, Helodidae and Dytiscidae are Coleoptera, Chironomidae and Ceratopogonidae are Diptera. Amphibian sloughs are from *Triturus alpestris* and *Bufo bufo*. *Z.* adj. = *Z.* adjusted.

[a] Large value due to only one individual.

of newts (*Triturus alpestris apuanus*, *Triturus vulgaris meridionalis* and *Triturus c. carnifex*). These characteristics support the hypothesis that the presence of varied underexploited microhabitats favours resource partitioning (Malmquist et al., 1992; Robinson et al., 1993; Skulason & Smith, 1995), but show that the presence of such heterogeneity is not an obligate prerequisite for the maintenance of polymorphisms. Alternative explications have thus to be found, particularly in the life-history of the newts coping with habitat uncertainty (Kalezic & Dzukic, 1985; Denoël & Joly, 2000; Denoël et al., 2002).

Apennine ponds are small aquatic habitats devoid of a vertical component, in contrast to Alpine lakes, which are often very deep (e.g. 7 m depth : Denoël & Joly, 2001a). Consequently, in a rather homogeneous environment, alternative phenotypes might profit less from their morpho-physiological status (e.g. trophic apparatus, gills, and body shape), even in the absence of newt competitors as is the case in the studied pond (Denoël et al., 2001b). In the absence of deep waters, paedomorphs have also no possibility to avoid competition with metamorphs in shallow waters. Differences in habitat and food use between

alternative morphs within a species have been shown in animal groups other than the amphibians. For instance, in the bluegill (*Lepomis macrochirus*), individuals differ in the size of their pectoral fins. This specialization gives them specific foraging advantages : in open water for the individuals with short fins and in vegetated areas for the individuals with long fins. The two morphs are also observed in the micro-habitat where they are expected (Ehlingher, 1990). However, in heterochronic newts, no morphological trait supports the higher abundance of paedomorphs in the vegetation area than in the open area. Indeed, paedomorphs would be particularly expected in open water. Such habitat preference might be connected to some foraging tactics because prey distribution differs between the two habitat components (M. Denoël, pers. obs.), but laboratory experiments are needed to test this hypothesis.

Paedomorphs and metamorphs possess a different feeding mechanism. In both morphs, prey items are sucked in with water, but metamorphs have to expel water out of the mouth after suction because their gill slits are closed (Joly, 1981; Lauder & Shaffer, 1993). These morphological differences lead to differences in efficiency of catching prey : paedomorphs are better predators on crustaceans whereas metamorphs are more efficient at catching terrestrial invertebrates (Denoël, 2001). Despite these characteristics, paedomorphs do not eat many more plankton (except in April in males) and less terrestrial invertebrates than metamorphs in the studied pond. In fact, although planktonic organisms were relatively abundant (around twenty individuals per litre) in the eutrophic pond, adults caught only a few individuals each day. This means that far fewer plankton were eaten than in Alpine lakes where plankton was an important element of the diet (around sixty planktonic individuals per paedomorph stomach : Denoël et al., 1999; Denoël & Joly, 2001a). This lack of utilisation of small prey might originate from the abundance of other prey in the diet of newts (e.g., chironomid larvae and newt eggs) (Joly, 1987a). Higher ingestion rates of chironomid larvae in paedomorphs than in metamorphs were also shown in experimental trials (Denoël, 2001). In the studied pond, terrestrial invertebrates were very rare, suggesting that metamorphs are not particularly specialized on this diet. On the contrary, in oligotrophic Alpine lakes, terrestrial invertebrates are abundant during the aquatic active period of the newts, and comprise a large part of the diet of the newts (Chacornac & Joly, 1985; Sattmann, 1989; Joly & Giacoma, 1992; Schabetsberger & Jersabek, 1995).

The diet of newts was not identical in March and April. These differences were mainly due to variation in prey abundance between these two months. For instance, the small number of newt eggs consumed in March was due to the oviposition period of the newts, which was more intense in April than in March. More amphibian sloughs were foraged in March as a consequence of the adaptation of the newts to their new environment (Griffiths, 1996) and to the presence of common toads *Bufo bufo* only in March. Large densities of prey might also increase resource overlap (Hindar & Jonsson, 1982; Griffiths, 1986; Smith, 1990), but similar food niche overlaps between morphs were found in March and April.

Eggs of newts are usually laid in the aquatic vegetation (Miaud, 1995), increasing their survival against invertebrate and vertebrate predators (Miaud, 1993, 1994). However, Alpine newts foraged particularly on this kind of prey. Considering fecundity of Alpine newts (a few tens to hundreds of eggs : Kalezic et al., 1996) and the large number of eggs eaten (more than one egg per newt per day in this study), this predation affects general productivity and potentially limits the population size. In the studied site, newt density was very high : more than 2000 newts for a 100 m^3 pond. This predation mechanism might then be regulated by population density. It is also mainly exhibited by females, as previously outlined by other authors (Joly, 1987a; Sattmann, 1989; Joly & Giacoma, 1992). Kin selection experiments suggests that some amphibian species avoid eating their own progeny (Blaustein & Walls, 1995). However, no kin selection was found in the smooth newt (Gabor, 1996), and the situation remains unknown in the Alpine newt.

Variation in body size, and thus in the gape width, can favour resource partitioning (Hutchinson, 1959). Numerous examples have been found in newts and salamanders (Leff & Bachman, 1986; Kuzmin, 1991; Joly & Giacoma, 1992), including in paedomorphic communities (Fasola & Canova, 1992; Denoël & Joly, 2001b). Paedomorphs from the studied site are younger and smaller than the metamorphs (Denoël & Joly, 2000). However, the difference in size (12 % in females, 7 % in males) does not appear to be related to predation tactics because the two morphs foraged on similar-sized prey. On the contrary, gilled juveniles largely differ from adults in diet use. They eat many more cladocerans and copepods than the adults, but also a few more insect larvae. The smallest gilled juveniles (23-34 mm) ate only small prey (mean prey length of about 1 mm), but the largest gilled juveniles behave more similarly to adults in eating different-sized prey (mean prey length of about 1 to 13 mm). Differences are related more to the size of individuals than to the acquisition of maturity. Mean prey size of adults was about 1 to 20 mm. There is thus an obvious resource partitioning between the two gilled immature classes and between gilled immature and adult stages. The avoidance of planktonic organisms by adults favours gilled juveniles, which are gape-limited. The limited trophic similarities between the gilled juveniles and the paedomorphs therefore favours the maintenance of paedomorphs in the ponds as the two have to coexist all the year round, in contrast to the metamorphs that leave the pond after breeding (M. Denoël, pers. obs).

In the studied site, paedomorphs gain fitness benefits from their earlier age at maturity (Denoël & Joly, 2000), but interfere with metamorphs in using similar dietary items and habitat components (this study). Once they mature in larval morphology, they can still gain advantages from resource partitioning but this benefit seems limited. However, paedomorphs gain high body condition and energy intake, which make the paedomorphic pathway advantageous in this habitat (Denoël et al., 2002) until the risk of the pond drying out and the high densities (12 individuals/m^2 in the studied pond) might make the site undesirable and then favour metamorphosis and dispersion of the paedomorphs (Denoël & Poncin, 2001; Denoël, 2001).

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Elevated salinities may enhance the recovery of hydrated heat-shocked *Artemia franciscana* cysts (International Study on *Artemia*. LXV)

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ABSTRACT. Hydrated encysted embryos of the crustacean *Artemia franciscana* Kellogg 1906 were incubated at 42ºC and 44ºC for 48 h at elevated salinities. They exhibited better recovery when incubation took place in a high salinity environment. The recovery and/or protection by salinity of *A. franciscana* encysted embryos depend on the severity of the heat shock. Cysts heat shocked at 44ºC and different salinity media (35, 50 and 70 ppt), upon return to optimal conditions, gave similar hatching percentages of less than 5%. When heat shock took place at 80 ppt, a significant increase in hatching was observed (i.e. from 5% to 22%). Concentration of glycerol and translocation of a major protein of about 26 kD (p26) in *Artemia* encysted embryos were studied to reveal the potential protective role of these two factors against elevated temperatures. Increased glycerol levels were scored when cysts were incubated in elevated salinities within the first 4 h and prior to heat shock treatment (i.e. a 38% and 49% increase at the salinities of 70 ppt and 80 ppt, respectively). SDS-PAGE of proteins extracted from control and heat-shocked cysts suggested that the intracellular translocation of p26 was enhanced as the salinity increased. It is proposed that salinity-dependent heat shock seems to be at least partly responsible for i) the significant increase of glycerol and ii) the degree of translocation of p26, a suspected molecular chaperone described previously. These two factors along with others may contribute to the better recovery of heat-shocked *Artemia* encysted embryos. This phenomenon, which may be attributed to increased thermostability of proteins, is reflected as different cyst hatching ability.

KEY WORDS : *Artemia franciscana*, salinity, heat shock, glycerol, p26.

INTRODUCTION

The brine shrimp *Artemia* (Crustacea, Anostraca) is the main zooplanktonic organism that inhabits hypersaline environments all over the world (Triantaphyllidis et al., 1998). This branchiopod has acquired extremely capable adaptive mechanisms to survive and evolve in habitats with extensive and often abrupt fluctuations in salinity, UV irradiation, temperature, and oxygen concentration (Persoone & Sorgeloos, 1980). These mechanisms are poorly understood, although several studies showed the ways that *Artemia* responds under various abiotic conditions prevailing in its natural habitats (for extensive review see Abatzopoulos et al., 2002 and references therein). Miller & McLennan (1988a) showed that metabolizing encysted embryos are more thermotolerant compared to 24 h-larvae, while thermotolerance can be induced in adult *Artemia* by the application of a sub-lethal heat treatment or a short period of heat shock, which results in the synthesis of heat shock proteins and heat shock related proteins (Miller & McLennan, 1988b). However, prolonged heat shock can be lethal (Miller & McLennan, 1988a).

Sorgeloos et al. (1976) first demonstrated that if fully hydrated cysts remain for 24 h at 40ºC, the embryos stop developing, but without any significant decrease in their survival upon return to a favourable temperature of 28ºC. However, repeated cycles of 8 h at 28ºC followed by 24 h at 40ºC lead to considerable decrease in the hatchability and viability of the embryos (Sorgeloos et al., 1976). Clegg et al. (1994) as well as Abatzopoulos et al. (1994) gave new insights into the effects of prolonged anoxia and heat shock (42ºC) on the viability of the encysted embryos. They demonstrated that diapause can be a reversible phenomenon since some of the cysts do not lose their viability after heat shock or anoxia, but return to a state of diapause, which can be terminated by the application of a diapause-deactivation treatment (i.e. desiccation – Abatzopoulos et al., 1994). Although there is evidence that high temperature and lack of oxygen, two very important abiotic factors, «push» metabolically active cysts back into diapause, little is known about the interaction with salinity, another abiotic factor with very significant role in the life history of *Artemia* (Abatzopoulos et al., 2002). The interactions among salinity, trehalose breakdown, accumulation of free glycerol in the cyst, internal osmotic pressure and osmotic rupturing of

the chorion, i.e. the necessary steps towards excystment, have been described by CLEGG (1962, 1964).

Trehalose and glycerol may act as protectors inducing tolerance to heat and/or salt shocks; this is well documented in *Saccharomyces cerevisae* (CARVALHEIRO et al., 1999). Glycerol has also been implicated in the thermoprotection of proteins and whole cells (CARVALHEIRO et al., 1999 and references therein). The combination of osmotic pressure (expressed by NaCl concentration) and temperature enclosed a synergistic effect (CARVALHEIRO et al., 1999). NaCl contributed with an effect fivefold that of temperature on the synthesis of both trehalose and glycerol. SUN et al. (1999) revealed that glycerol stabilized an enzyme, glutamate dehydrogenase, against thermoinactivation. Moreover, their results showed that stabilization, which was induced by glycerol, increased with temperature. Glycerol had the same effects in thermostabilization as pressure (SUN et al., 1999) while it appears to be excluded from protein surfaces by the solvophobic effect (SUN et al., 1999 and references therein). However, glycerol is not completely absent from the surface of the proteins. In fact, glycerol has an affinity for polar regions of proteins (SUN et al., 1999 and references therein) and binds to proteins in direct proportion to its concentration. Glycerol has been shown to reduce the apparent specific volume of proteins in aqueous solutions and to decrease both the volume and compressibility of protein interiors. Compression of the protein structure has been rationalized through the reduction of voids or cavities within the protein (SUN et al., 1999). Also, according to CHEN et al. (2000), glycerol provided protection to IgG proteins from high temperatures by preventing denaturation or unfolding.

Another important factor that may have a similar role to glycerol is p26, a potential chaperone which is a small heat shock/α-crystallin protein and has been described in *Artemia* embryos (LIANG & MACRAE, 1999). A possible mechanism for chaperone action is to prevent irreversible aggregation and allow proteins to refold either spontaneously or with the assistance of other chaperones, through the interaction with exposed hydrophobic regions (see LIANG & MACRAE, 1999 and references therein). The association of p26 with the nucleus and its ability to protect organisms from thermal stress may have a key role during encystment, diapause and quiescence in *Artemia* embryos (LIANG & MACRAE, 1999).

Unfortunately, there is no published work on measurements of cyst temperatures under natural conditions, although it can be safely assumed that cysts (desiccated or hydrated) encounter thermal stresses when they are washed onto shore and exposed to sunlight warmth or to freezing temperatures (CLEGG & TROTMAN, 2002). MILLER & MCLENNAN (1988a) showed that hydrated cysts from Great Salt Lake, Utah (i.e. *A. franciscana*) were reasonably tolerant to high temperatures (LT_{50} was 49ºC for 1 hour exposures); they also demonstrated the presence of several isoforms of Heat Shock (HS) proteins belonging to HSp-70 and -90 families in cysts as well as a small HS protein (MILLER & MCLENNAN, 1988b). When hydrated cysts were exposed to temperatures in the range of 42-50ºC, a nuclear translocation of p26 took place (ABATZOPOULOS et al., 1994; CLEGG et al., 1999).

CLEGG et al. (2001) reported on the thermal tolerance and the HS proteins in *Artemia* cysts from widely different thermal habitats; they found that hydrated SFB cysts cannot stand 50ºC for more than 60 minutes (hatching percentage dropped to 0) while the higher thermal tolerance was observed in Vietnam *A. franciscana* cysts (hatching percentage dropped to 0 when these cysts were incubated at 50ºC for 90 minutes). Maximum water temperatures in San Francisco Bay during summer and spring very rarely exceed 24ºC while the Vietnamese *A. franciscana* strain experiences daily water temperatures near to 38ºC for most of the growing season (CLEGG et al., 2001 and references therein).

The correlation between heat shock and salinity has not been studied in *Artemia*. This paper describes how salinity affects the response of *Artemia* encysted embryos to prolonged heat shock and discusses the way salinity protects these early developmental stages by enhancing their thermotolerance or thermostability.

MATERIAL AND METHODS

Cysts of the bisexual species *A. franciscana* (from San Francisco Bay – SFB – USA, batch no. 65034, ARC code no. 1258) were used since they exhibited high hatching percentage and good hatching synchrony (ABATZOPOULOS et al., 1994). The number of dehydrated cysts per gram for this specific strain was 275,000 approximately.

Experimental design

The rationale for the experiments to follow (step by step) will be helpful to the reader. The experimental design included the following steps : (a) hydration of the cysts at optimal conditions for $1^1/_2$ h, (b) transfer of fully hydrated cysts to different salinities for $2^1/_2$ h under optimal conditions, (c) incubation of hydrated cysts in all different salinities at 42ºC and 44ºC, (d) cysts hatching under optimal conditions. During step (a), complete hydration of cysts is accomplished. In step (b), cysts are allowed to build up different glycerol levels/concentrations due to incubation in elevated salinities (CLEGG, 1962; 1964). During steps (a) and (b) the encysted embryos are in full metabolical stage (for determination of metabolism see ABATZOPOULOS et al., 1994). In this fully metabolizing condition the cysts were heat shocked for 48 h (in different salinity media – step (c)). In step (d), hatching ability of the cysts was estimated i.e. the percentage of cysts that hatched (cysts in quiescence) *versus* cysts in diapause. Determinations of water content, glycerol level, hatching under different conditions and translocation of p26 were performed.

Hydration of the cysts

Dry cysts were weighed out (0.2 g for determination of hatching percentage, 0.15 g for water content estimation and 0.5 g for glycerol determination; these weights were used for each replicate for each treatment – see below) hydrated for 90 min at 30 ppt medium prepared by diluting filtered brine of 140 ppt from the nearby saltworks of M. Embolon (Thessaloniki, Greece); maximum hydration for this SFB cyst material is achieved within this period.

Fully hydrated cysts were harvested on a sieve and placed in small cylindroconical glass tubes that contained 50 ml filtered water of various salinities i.e. 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 100 ppt (three replicates for each salinity and heat shock temperature). For the next $2^1/_2$ h, the temperature was 28ºC while light intensity 2000 lux and aeration were provided for the rest of the experiment. Then, the glass tubes containing the cysts were placed in water baths at 42±0.2ºC and 44±0.2ºC for 48 h while continuous aeration was applied in each tube. All tubes were carefully closed with parafilm and were frequently monitored for salinity, which remained constant.

Cysts water content

Once the prolonged heat shock treatment was completed, the cysts were harvested by rapid filtration and rinsed thoroughly with deionised water to remove salts; rinsing was a very rapid procedure i.e. no more than 10 sec; in this way, water uptake by the cysts was avoided. Then, 0.15 – 0.20 g of the heat-shocked cysts were placed in Eppendorf tubes (three or four for each of the scored salinities) bearing tiny holes at the bottom and centrifuged for 5 min at 13,000 rpm in order to remove water. In this way the cyst surface was free from salts or water. Afterwards the cysts were placed in pre-weighed cups and oven dried at 58±1ºC for 48 h.

Determination of hatching percentage after heat shock treatment

Cysts that were heat shocked at 35, 50, 70, 80 and 100 ppt (0.08 g per cylindroconical tube) were incubated under optimal conditions and the hatching percentage was determined according to Sorgeloos et al. (1986) at the end of 48 h (three samples of 250 µl each, were taken from each replicate and for each salinity treatment and in each of the two HS temperatures); although our final hatching data are reported for 48 h, we observed no increase in hatching levels after five days of incubation. Fully hydrated (non heat-shocked) cysts were incubated under optimal conditions at five different salinities (i.e. 35, 50, 70, 80 and 100 ppt) for 240 h (10 days) in order to exclude the case of a delayed hatching rate (Abatzopoulos et al., 1994).

Glycerol

Fully hydrated cysts (0.5 g) were placed at 35, 70 and 80 ppt (for each salinity, three samples were taken). Glycerol determination was performed according to the calorimetric method of Burton (1957) and following the procedures described by Clegg (1962, 1964).

SDS-PAGE

Denaturing polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed following Abatzopoulos et al. (1994). Control and heat-shocked (at 44ºC) cysts were homogenized (0ºC) in buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 0.1 mM phenylmethylsulfonyl fluoride, 40 mM Hepes, pH 7.55) using glass homogenizers (100 mg wet weight embryos/ml buffer K) and the homogenate centrifuged at 1630 g (4ºC) for 5 min following Clegg et al. (1994). The resulting pellets (which contained nuclei, yolk platelets and shell fragments) were washed with 50 times their volume in buffer K and resuspended to their original volume. Aliquots of pellets and supernatants were added to 2x sample buffer (Laemmli, 1970) heated at 100ºC for 5 min, and centrifuged at 10,000 g for 10 min. The supernatants were analyzed on 15% SDS polyacrylamide gels and proteins stained with Coomassie brilliant blue G-250 (CBB : 0.25 w/v in methanol 40% v/v and acetic acid 8% v/v). Quantification of relative amounts of p26 was evaluated densitometrically.

Statistical analysis

The variables analysed were : a) the cyst water content in various salinities, b) the hatching percentage of heat-shocked cysts at 42ºC and different salinities, c) hatching percentage of heat-shocked cysts at 44ºC and different salinities. Statistical analysis was performed using parametric (ANOVA) and non-parametric (Kruskal-Wallis) statistical tests. The selection of the statistical test was based on the assumption that the variances were homogeneous (Sokal & Rohlf, 1981). Bartlett's, Hartley's, Cochran's and Levene's tests were applied to determine the homogeneity of the variance. STATISTICA 5.1 and STATGRAPHICS 5.0 were used for the analyses.

RESULTS

Water contents of cysts in various salinities after incubation for 48 h at 42ºC are presented in Table 1. The water content varied from about 62% at 35 ppt to about 54% at 100 ppt. Comparison of the cyst water contents revealed that there were significant differences among the examined salinities (ANOVA, $F = 3.207$, $df = 10, 29$, $p < 0.05$). Salinities between 35 ppt and 70 ppt resulted in similar cyst water content while apparent differences existed between the water content in 35 ppt and 75 ppt onwards (Duncan's multiple range test, $p < 0.05$ – see Table 1). The same battery of experiments performed at 44ºC, exhibited no differences in cyst water content compared to those at 42ºC (Kruskal-Wallis, $p > 0.05$) and, therefore, are not presented here.

TABLE 1

Water content in *A. franciscana* cysts (expressed as percent of the dry cysts, w/w) after 48 hours incubation at 42˚C in various salinities. Results that share the same letters are not significantly different (Duncan's multiple range test, $p < 0.05$). n : number of samples.

Salinity (ppt)	Water content (%)	n
35	61.69 ± 0.85 (a)	4
40	60.89 ± 1.04 (a,b)	3
45	60.27 ± 1.11 (a,b)	4
50	59.02 ± 1.94 (a,b)	3
55	59.07 ± 1.49 (a,b)	4
60	59.29 ± 1.01 (a,b)	3
65	59.51 ± 1.14 (a,b)	4
70	58.33 ± 2.23 (a,b)	4
75	57.27 ± 4.71 (b,c)	4
80	56.92 ± 2.44 (b,c)	3
100	54.07 ± 3.21 (c)	4

Hatching percentages (HP) of the heat-shocked cysts (42ºC) in each salinity presented significant differences (ANOVA, $F = 20.78$, $df = 4, 14$, $p < 0.05$ – see Table 2). A significant drop in the apparent hatching capability occurred in the heat-shocked cysts (42ºC) at salinities below 70 ppt (i.e. from 92.5% in control material, decreased to below 70%, Scheffé test, $p < 0.05$, see Table 2). Cysts that were heat shocked at 44ºC in the examined salinities presented significant differences in their HP upon their return to optimal hatching conditions (ANOVA, $F = 343.86$, $df = 4, 14$, $p < 0.05$ – see Table 2). The drop of the HP at this temperature was dramatic (i.e. from 92.5% – see heading of Table 2 – decreased to less than 5% when HS salinity was 70 ppt, Scheffé test, $p < 0.05$, see Table 2). It seems that at 44ºC there was a threshold between the salinities of 70 ppt and 80 ppt; cysts that were heat shocked at the salinity 80 ppt and above showed a higher level of recovery (Scheffé test, see Table 2). Cysts that were incubated in 100 ppt during heat shock at 44ºC, resulted in a hatching percentage 25 times higher than those incubated in 35 ppt during heat shock (i.e. 41.72% compared to 1.67%, Scheffé test, $p < 0.05$, Table 2). Therefore, at 44ºC the hatching percentage of heat-shocked cysts in the respective salinities (Table 2), revealed significant differences upon return to favourable conditions. In this way, they exhibited a clear trend of higher recovery capability as the salinity, in which the prolonged heat shock took place, rose.

TABLE 2

Hatching percentage (HP) of *A. franciscana* cysts after 48 h incubation at 42˚C and 44˚C in 5 different salinities and returned to optimal conditions (HP was determined after 48 h). Results that share the same letters in each column are not significantly different (Scheffé test, $p < 0.05$). The number of samples measured for each treatment (temperature and salinity) was 3 (3 subsamples were taken per replicate). Cysts incubated at optimum conditions (S : 35±2 ppt, T : 27±1ºC, pH = 8.75) without a prior heat shock (control) resulted in a hatching percentage of 92.50±1.88%.

Salinity (ppt)	Hatching percentage (HP, %)	
	After heat shock at 42°C	After heat shock at 44°C
35	66.45 ± 2.90 (a)	1.67 ± 1.07 (a)
50	68.69 ± 2.87 (a)	2.35 ± 1.45 (a)
70	68.91 ± 3.16 (a)	4.09 ± 2.73 (a)
80	70.40 ± 2.31 (a)	22.37 ± 3.64 (b)
100	77.18 ± 2.03 (b)	41.72 ± 4.01 (c)

When cysts not subjected to heat shock were incubated in five different salinities (i.e. 35, 50, 70, 80 and 100 ppt – the rest of the conditions were at optimal levels), the following results were observed : when salinity increased above 70 ppt, there was a delay in hatching rate while cysts incubated in 100 ppt presented less than 5% hatching after 8 or 10 days denoting that a minimum metabolism was taking place (Fig. 1). The rationale of this experiment is discussed in the discussion section.

Fully hydrated cysts placed in different salinities (35, 70 and 80 ppt) revealed different glycerol levels

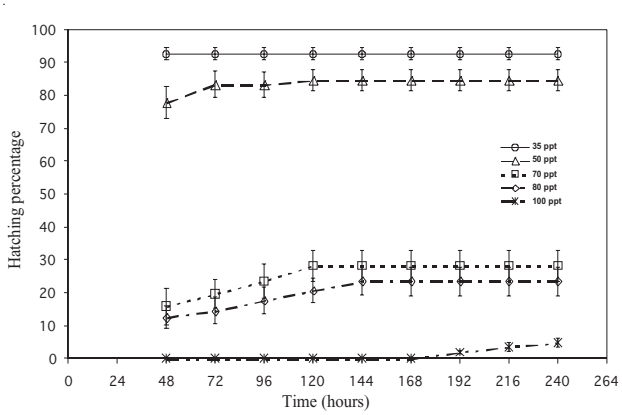

Fig. 1. – Hatching percentage (HP) of *Artemia franciscana* cysts in various salinities (all other hatching conditions were kept optimal).

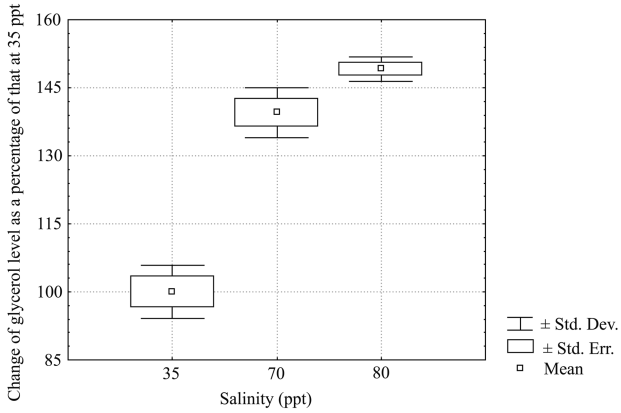

Fig. 2. – Changes in glycerol levels with increasing salinity. At 35 ppt, glycerol levels were arbitrarily considered as 100%.

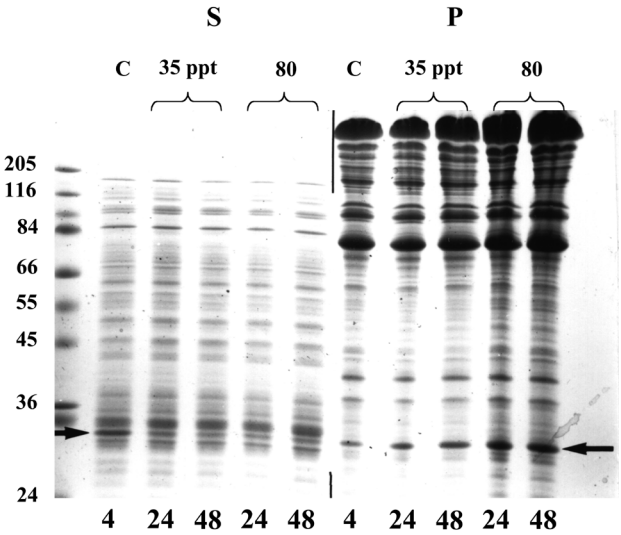

Fig. 3. – SDS-PAGE profiles of fractions from hydrated but non-heat-shocked cysts of *Artemia franciscana* (4), and after 24 h and 48 h at 42˚C in 35 ppt and 80 ppt. Supernatant (S) and pellet (P) fractions were analyzed (see Materials and Methods). Molecular weight standards are in kiloDaltons (kD). The arrow denotes the 26 kD protein.

after 2 1/2 h (Fig. 2). Glycerol levels at 35 ppt were arbitrarily considered as 100 (±5.96)%. When salinity increased from 35 ppt to 70 ppt, the glycerol concentration increased by 39 (±5.49)%, while in 80 ppt the increase was nearly 50% (49.5±2.73%). These differences in glycerol levels were obviously significant (Kruskal-Wallis, $H = 7.2$, $n = 9$, $p < 0.05$).

SDS-PAGE of hydrated *Artemia* cysts not exposed to heat shock treatment and heat shocked at 44ºC for 24 and 48 h in 35 ppt and 80 ppt revealed very similar protein profiles (see Fig. 3). There is, however, one exception : a protein of about 26 kD (p26) appears to be translocated from the supernatant (S, soluble fraction) to the pellet (P, mainly nuclei) as a result of heat shock. Moreover, the amount of p26 in the pellet fraction from 80 ppt was fairly increased compared to that from 35 ppt.

DISCUSSION

Incubation of fully hydrated cysts in various salinities at 42 or 44ºC for 48 h and immediate transfer to optimal conditions for determination of hatching percentage, showed a better recovery of the cysts that were heat shocked in a high salinity environment. There is a gradual increase in hatching percentage as salinity elevates. Even a small increase in salinity of the order of 10 ppt (i.e. from 70 ppt to 80 ppt) seems to offer the embryos a substantial advantage against the stressor of 44ºC (i.e. hatching percentage was increased from 4.09% to 22.37% – Table 2). This advantage offered by the elevated salinity to *Artemia* cysts, raises several questions regarding the mechanisms that induce this phenomenon. One explanation would be that the various salinities affect the hydration level of the cysts, hence, their metabolic status and sensitivity to various shocks. This seems not to be valid since in this work, the results from the water content determination (see Table 1) suggested that the hydration level of the cysts was sufficient to sustain metabolism in the encysted embryos for nearly all salinities tested with the exception of 100 ppt, as referenced by the hatching response (see Fig. 1). These results are consistent with the opinion that above salinity levels of 90 ppt, which is a potential "threshold" for most *Artemia* populations, water is absorbed in insufficient quantities to support the metabolism of the embryos and consequently they remain in quiescence (Lavens & Sorgeloos, 1987) or exhibit a very retarded hatching (see incubation at 100 ppt – Fig. 1).

It is well known that salinity is one of the major factors that influences the hatching ability of *Artemia* cysts by affecting the osmotic potential of the embryo (Conte et al., 1977) and special biochemical activities such as that of the hatching enzyme (Sato, 1967). The salinity of the incubation medium affects the water content of the cysts and thus the accumulation of free glycerol (Clegg, 1964); gradual accumulation of the glycerol in the cyst causes a corresponding increase in the internal osmotic pressure. This enhances the ability of the cyst to imbibe water and therefore to continue its development; then the chorion of the cyst ruptures osmotically only if the increased internal osmotic pressure eventually exceeds that of the environment (Clegg, 1964).

As the water content is obviously not the only parameter responsible for the metabolic status of the encysted embryos or their behaviour during incubation at high salinities and temperatures, the question arises about the exact role of salinity in the recovery of cysts from heat shock. Three other factors may play an important role in cyst thermostability : glycerol, p26, trehalose or combinations of these (Clegg & Trotman, 2002). Only two of them (i.e. glycerol and p26) are involved in heat protection of hydrated cysts, while trehalose is related to desiccated cysts (Crowe et al., 2001 and references therein). Therefore, the question addressed above may be reconstructed as follows : In what way does salinity enhance the tolerance of fully hydrated cysts to heat shock temperatures? Is it due to glycerol concentration and/or translocation of p26?

Glycerol concentration

The heat-shocked cysts at 42ºC presented a slight but statistically different reduction of the hatching percentage compared to the control cysts; this was probably due to the percentage of the cysts re-entering diapause (Abatzopoulos et al., 1994). There were no differences in the hatching percentages of the heat-shocked cysts in different salinities with the exception of the outcome in 100 ppt (see Table 2). At 44ºC, the hatching percentage of the heat-shocked cysts reduced significantly compared to the control cysts, while a relationship between the elevation of salinity and the hatching percentage appeared (hatching percentage of the heat-shocked cysts in 100 ppt was 25 times higher than the heat-shocked cysts in 35 ppt – see Table 2). The dramatic drop in hatching percentage at the low salinity treatment must be attributed to cysts that either enter a deep diapause (from which we cannot recover them with the empirical techniques presently at our disposal, see Van Stappen et al., 1998) or die.

It seems that there is a "threshold" in salinity levels; cysts that were heat shocked in 80 ppt or 100 ppt hatched upon return to optimal conditions (hatching percentage : 22.37±3.64% and 41.72±4.01%, respectively). One explanation would be based on the water content of the cysts and glycerol's properties (see Introduction – Sun et al., 1999 and references therein). Heat-shocked cysts in relatively low salinities (35 ppt to 70 ppt) contained more water than those in higher salinities (80 ppt or 100 ppt). This reduction of water content (about 14% in 100 ppt) was one of the parameters that caused the increase of glycerol concentration. The other parameter was the build up of cyst glycerol (for increasing internal osmotic pressure) in elevated salinities towards rupturing of chorion (step b of the Experimental design). It is obvious (see Fig. 2) that the salinity increase is related to the increase of the concentration of glycerol (corresponding to an increase of almost 50% in 80 ppt compared to 35 ppt). Thus, these two incidents must be responsible for the substantial increase of glycerol concentration and most probably for the protection of proteins (see Introduction). As a consequence, heat-shocked cysts in the high salinity treatment showed better hatching compared to those treated in low salinities, since the latter were driven either to re-enter diapause or to "die".

p26

The higher levels of p26 in nuclei that were observed after prolonged heat shock in 80 ppt compared to those in 35 ppt, is an indication that this salinity level activates translocation of the molecular chaperone p26, which protects cysts from heat shock. Previous work of Abatzopoulos et al. (1994) has demonstrated that after 48 h heat shock at 42ºC and 35 ppt salinity, at least some *Artemia* cysts are pushed back into a diapause state. This diapause induction was accompanied by a translocation of a 26 kD protein from the supernatant to the pellet (i.e. from the cytoplasm into nuclei). Clegg et al. (1994) and Liang et al. (1997a,b) demonstrated that anoxia and prolonged incubation at low temperatures (0-20ºC, one month) can also induce a similar translocation, the same as seen in embryos exposed to aerobic heat shock (42ºC) (Abatzopoulos et al., 1994). In this study, a major translocation of p26 was not apparent, although a gradual increase of p26 was recorded in the pellet from 80 ppt and 44ºC. Clegg et al. (1996) showed that encysted embryos in the state of diapause produced in laboratory cultures and collected from field, also share the p26 between the fractions of pellet and supernatant. Clegg et al. (1994) presented evidence that the 26 kD protein is a subunit of a native protein with molecular mass of about 500 kD (or 700 kD according to more recent work – Jackson & Clegg, 1996; Liang et al., 1997a, b), and that its role might be that of a protective molecular chaperone (Jackson & Clegg, 1996). Our data revealed that both salinity and heat shock seem to have an additive effect, since the intensity of the 26 kD protein in the pellet after 24 h and 48 h heat shock at 44ºC and 80 ppt salinity is higher when compared to a similar heat shock in 35 ppt. The above results are in agreement with the hypothesis of Clegg et al. (1994) that the 26 kD protein might be a protective molecular chaperone, since it allows the embryos to withstand elevated temperatures without a tremendous loss of their hatching ability.

Concluding, it is evident that elevated salinities result in increased glycerol concentrations, which, together with increased p26 in nuclei, may prevent the encysted *Artemia* embryos from entering a deep diapause state from which they cannot be recovered by applying empirical techniques. The major role of glycerol as thermostabilizing agent has to be stressed although further experimentation is needed to document this hypothesis.

Attempting to explain an ecological significance of this phenomenon, one may attribute it to the special conditions that cysts face when washed ashore. It has been observed quite frequently and by many teams (personal communication) that cysts – when driven ashore – float in shallow waters (the water column is only few cm) with salinities ranging from 80-200 ppt and water temperature as high as 45ºC or more. Since this situation is very common, this phenomenon may be of high adaptive significance for *Artemia* encysted embryos. These findings have been confirmed in other *Artemia* populations, such as parthenogenetic populations (unpublished data).

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Sexual dimorphism and morphometric variability of cheek teeth of the cave bear (*Ursus spelaeus*)

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ABSTRACT. The sexual dimorphism and the morphometric variability of the cheek teeth in *Ursus spelaeus* from six geographically well-separated localities dating from the Middle Weichselian were studied. The sexual dimorphism of the canines and of the lower carnassials (m1) of the cave bear are as much or more expressed than the dimorphism of these teeth in the Recent brown bear. The examined cave bear assemblages are rather similar in tooth size and proportions. The differences between the assemblages were presumably influenced by the ratio of male to female bears. The posterior cheek teeth M2 and m3 allowed us to divide more northern (Goyet in Belgium, Niedzwiedzia in Poland, and Medvezhiya Cave in European Russia) from more southern (Eirós in Spain, Arcy-sur-Cure in France, and Odessa in Ukraine) sites. These grouping suggest a difference in the diet of the cave bears in the northern and southern parts of the species distribution range, at least during the time segments studied.

KEY WORDS : *Ursus spelaeus*, cheek teeth, morphometric variability, sexual dimorphism, geographical variability

INTRODUCTION

During the Last Glacial, the cave bear (*Ursus spelaeus* Rosenmüller, 1794) was widely spread in Europe from the Atlantic coast to the Ural Mountains. The nature of its geographical variability is not clear. According to VERESHCHAGIN & BARYSHNIKOV (1984), every karst region possessed its own local population of *U. spelaeus*. The cheek teeth from successive stratigraphic levels are suitable for elaborating a model of the evolution of the dental system (RABEDER, 1983, 1999; BARYSHNIKOV, 1998). However, are cheek teeth appropriate for the analysis of the geographic variation of *U. spelaeus*? Does the sexual dimorphism vary in different cave bear groups? We have raised these questions in studying the dental collections from several sites.

LOCALITIES, MATERIAL AND METHODS

Cave localities and collections :

AsC : Arcy-sur-Cure, France (c. 37,5-31,8 Ka BP, GIRARD et al., 1990), collections of Institut de Paléontologie, Paris, France

CE : Cova Eiros, Spain (c. 28,2 Ka BP, GRANDAL D'ANGLADE, 1993), collections of Instituto Universitario de Xeoloxia, Universidade da Coruña, Spain

GB4 : Goyet, Belgium, assemblage B4 (c. 35,5 Ka BP, GERMONPRE & SABLIN, 2001), collections of the Royal Belgian Institute of Natural Sciences, Brussels, Belgium

Ni : Niedzwiedzia, Poland (< 30 Ka BP, NADACHOWSKI et al., 1989), collections of the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Krakow, Poland

Me : Medvezhiya, Russia (cultural layers : c. 12,0 Ka BP, SINITSYN & PRASLOV, 1997; cave bear bone : > 48,6 Ka BP, RABEDER, personal communication), collections of the Zoological Institute, Russian Academy of Sciences, Saint-Petersburg, Russia

Od : Odessa, Ukraine (c. 26,9 Ka BP, KURTÉN, 1969), collections of the Zoological and Geological Museum, University Helsinki, Finland; the Zoological Institute, Russian Academy of Sciences, Saint-Petersburg, Russia; the Palaeontological Institute, Russian Academy of Sciences, Moscow, Russia

Three sites are located near the northern limit of the cave bear range : Goyet in Belgium, Niedzwiedzia in Poland and Medvezhiya in the Ural Mountains (Russia). Three others occur markedly to the south : Cova Eirós in Spain, Arcy-sur-Cure in France and Odessa in Ukraine. The morphometrical data on the cheek teeth from Arcy-sur-Cure were published earlier (BARYSHNIKOV & DAVID, 2001). The collection from Arcy-sur-Cure here studied is from the Upper Palaeolithic layers of grotto Renne.

The cave bear teeth were measured using the scheme published earlier (BARYSHNIKOV, 1998; BARYSHNIKOV & DAVID, 2000). The measurements were taken with dial calipers with accuracy up to 0.1 mm. Heavily worn teeth were not measured. The data were processed by Cluster Analysis from STATISTIKA 6.0 (> 1999 edition).

LIST OF ABBREVIATIONS

BL : basal length,
CBL : condylobasal length,

CV : coefficient of variation,
DtC : transversal diameter canine,
F : Female,
Fm : Female mean,
GL : greatest crown length,
Gln : greatest length of nasals,
GWocc. : greatest width of the occipital condyles,
GW : greatest crown width,
LaLTa : labial length of talonid,
LaLTr : labial lenght of trigonid,
Lcp : length of caudal part,
LE1 : Length of entoconid 1,
LE2 : length of entoconid 2,
Lfp : length of frontal part,
LiLTa : lingual length of talonid,
LiLTr : lingual length of trigonid,
LMe : length of metacone,
LP4-M2 : length of maxillary tooth row P4-M2,
LPa : length of paracone,
LTa : length of talonid,
LTr : length of trigonid,
M : male,
Mm : male mean,
m : mean,
MLPC : minimal length between frontal ridge of protocone and caudal side of crown,
MW : minimal width,
Mws : minimal width of the skull,
OR : observed range,
P : level of reliability
SD : standard deviation,
t : t-test, Student criterium of reliability,
W C : width at the canine,
Why : width of tooth through hypocone,
WTa : width of talonid,
WTr : width of trigonid,
WZ : zygomatic width,

SEXUAL DIMORPHISM

As demonstrated by Kurtén (1955, 1976), the sexual dimorphism of cave bears is well marked in the size of the upper and lower canines, and cave bear males were considerably larger than females. Therefore, differences in canine and bone mean dimensions from various sites may depend on the different ratio between males and females. The cave bear assemblage B4 from Goyet contains several skull fragments of which more than half belong to males. Measurements of the skulls are given in Table 1.

One of these skulls (no. 2201), judging from its basal length, approaches the maximum size known for *U. spelaeus* (Cordy, 1972). The preponderance of male bears was also observed in the length of the mandibular tooth row p4-m3 and in the distribution of the canines (Germonpre & Sablin, 2001). In five male mandibles the length of the p4-m3 ranges between 107.2 and 115.5 mm with an average of 110.8 mm, in three female specimens the range is : 92.2-102.0 mm, and the average is : 97.5 mm.

The sexual dimorphism of the width of the lower canine was calculated as the ratio of male mean to female mean (Van Valkenburgh & Sacco, 2002). The dimorphism obtained for isolated canines from Goyet B4 is 1.28 (n M : 34, n F : 22; Germonpré, unpublished data), the mean for Odessa, based on the mean of female and male canine width given in Kurtén (1976), is 1.29. Both

TABLE 1

Measurements of the skulls of *Ursus spelaeus* from Goyet (assemblage B4), Belgium

Male					
	n	**OR**	**m**		
BL	3	409.0-445.0	428.0		
GLn	2	98.0-121.0	109.5		
LP4-M2	3	90.8-94.9	93.2		
GWocc	1	76.2	-		
WZ	-	-	-		
MW	1	98.7	-		
W C	2	102.8-114.8	108.8		
Female					
	n	**OR**	**m**	**SD**	**CV**
BL	2	372.0-390.0	381	-	-
GL n	1	80.9	-	-	-
L P4-M2	4	83.3-93.1	88.8	4.08	4.60
GW occ	2	79.3-84.8	82.5	-	-
WZ	1	206.0	-	-	-
MW	1	72.6	-	-	-
W C	2	83.0-99.0	91.0	-	-

are much higher than the mean of 1.13 for recent brown bear and are comparable to the mean of 1.25 for recent lion and 1.24 for recent leopard (data from Gittleman & van Valkenburg, 1997). According to these authors, dimorphism in canine size is related to severe male-male competition and frequent incidence of infanticide in polygynous species. These behaviours were therefore probably at least as much pronounced in cave bear as in Recent brown bear. According to Kurtén (1955), sexual dimorphism is stronger in cave bears than in Recent brown and polar bear. Furthermore, the dimorphism becomes more accentuated with increasing size.

The carnassials of bears show a weak dimorphism. The mean sexual dimorphism of the lower carnassial for brown bear equals 1.04 (Gittleman & van Valkenburg, 1997). The same ratio is obtained for two Russian subspecies of the brown bear : *Ursus arctos arctos* Linnaeus, 1758 from the north of European Russia and *Ursus arctos piscator* Pucheran, 1855 from Kamchatka (Table 2). However, in the subspecies *Ursus arctos pruinosus* (Blyth, 1854) from Tibet, the sexual dimorphism of the carnassial is much more expressed with a value of 1.09. Our data indicate that male and female brown bears reliably differ in means of the m1 length. The values of t-test change from 1.95 in *Ursus arctos arctos* ($P<0.05$) to 5.78 in *Ursus arctos pruinosus* ($P< 0.001$) (Table 2). The difference between male and female carnassial length is more pronounced for those brown bears that possess larger teeth, as shown by the Tibet sample. Although the first molar from the Kamchatka bears is quite large, sexual dimorphism remains small. Possibly the feeding by both males and females of these bears on soft and nutritious food, containing a large amount of salmon (Revenko, 1993) does not require tooth enlargement, even in large males. Presumably the sexual dimorphism in cheek teeth size in bears is allometric in nature and is revealed only when the tooth size exceeds a threshold level. This is probably reached in the Tibet bears, which

TABLE 2

Greatest crown length of the lower carnassial of male and female Recent *Ursus arctos* and of the lower carnassial in sexed jaws from *Ursus spelaeus* from Goyet (assemblage B4)

Samples	sex	n	OR	m	t	P	SD	sex dimor.	Mm-Fm	MSD/FSD
Ursus a. arctos										
European Russia	M	21	20,9-26,4	23.41	-	-	1.50	-	-	-
	F	18	20,5-25,0	22.55	-	-	1.26	-	-	-
	M+F	50	20,5-26,4	22.97	1.95	<0,05	1.43	1.04	0.86	1.19
Ursus a. piscator										
Kamchatka	M	15	24,1-27,9	25.89	-	-	1.23	-	-	-
	F	13	23,5-25,8	24.80	-	-	0.64	-	-	-
	M+F	30	22,8-27,9	25.28	2.99	<0,01	1.19	1.04	1.09	1.92
Ursus a. pruinosus										
Tibet	M	16	25,1-28,1	26.41	-	-	0.83	-	-	-
	F	7	23,4-25,9	24.14	-	-	0.88	-	-	-
	M+F	23	23,4-28,1	25.72	5.78	<0,001	1.35	1.09	2.27	0.94
Ursus spelaeus										
Goyet B4	M	10	28,7-32,6	30.92	-	-	1.23	-	-	-
m1 in sexed jaw	F	8	27,3-29,6	28.29	-	-	0.90	-	-	-
	M+F	18	27,3-32,6	29.75	5.23	<0,001	1.72	1.09	2.63	1.37

feed on rough plant material (Zhiryakov & Grachev, 1993). The sexual dimorphism of the Recent brown bears vary in the different geographical groups of the brown bear. Since the teeth of the cave bear from Goyet are larger than those of the modern brown bear, the difference in mean tooth lengths between males and females might be more considerable than in the Recent brown bear. The sexual dimorphism of the crown length of lower carnassials from the cave bears of Goyet was calculated, based on the carnassials in the sexed jaws (Table 2). The jaws were defined as male or female judged on the size of the canine. The sexual dimorphism of the sexed sample is 1.09, comparable with the dimorphism of the Tibet brown bears, and larger than that of the other brown bears. The value of the t-test is 5.23, the two-tailed P-value is less than 0.001, the difference between the male and female carnassial length from Goyet is extremely statistically significant (Table 2). The sexual dimorphism of the m1 of the cave bears from Mixnitz, Austria, is comparable with a value of 1.08 (data from Kurtén, 1955, table 8). Furthermore, the fact that the dimorphism of the canine is larger than that of the carnassial indicates the influence of the breeding system on the canine size rather than that of the feeding process (Gittleman & Van Valkenburg, 1997).

The carnassial mean length of the sexed subsample from Goyet (m1 in situ) is 29.75 mm. This subsample is probably more balanced (n M : 10, n F : 8 - males : 56%) than the total sample from Goyet. The mean male frequency of the Goyet assemblage is 69%, based on the third incisors, canines, skulls and lower jaws present in assemblage B4 (Germonpré & Sablin, 2001). However, this mean is based on adult and subadult specimens and the frequency of the males could be different in the sample of the lower carnassials as it contains a large frequency of young animals (Germonpré, in press). The mean of the more balanced subsample is much smaller than the mean crown length of all lower carnassials, isolated and attached in the lower jaw, from Goyet, which is 30.95 mm, and than the mean crown length of the other studied assemblages as well (Table 3) (Tables 3-9 : see appendix). Different causes may explain the discrepancy between the mean lengths of the lower carnassials from all studied assemblages, all being assigned to the Pleniglacial. The variation among the crown length means is statistically significant (P=0.0373).

The discrepancy may be due to :

1. The different ratio between the males and females in the assemblages
2. Individual peculiarities of the samples. They may depend on : (a) the number of specimens in each sample, which could be too small to show a real mean, (b) the individual variability of cave bears, (c) the individual age of the specimens as natural selection might accumulate unsuccessful variants among young animals, etc.
3. Different diet of the populations (see further)
4. Different system of taking measurements.

According to Grandal d'Anglade (2001), a predominance of males in a cave bear population would lead to an increment in the average values of the cheek teeth, while a preponderance of females would decrease the average. The large frequency of males in assemblage B4 from Goyet is explained by sexual segregation (Germonpre, *in press*). According to the frequency distribution of the lower canines, the assemblage from Odessa shows almost the same presence of males and females (Kurtén, 1976). The caves of Arcy-sur-Cure, Eirós, Niedzwiedzia and Medvezhiya Cave were probably used as dens predominantly by females. The abundance of deciduous bear teeth and the larger frequency of female canines in Arcy-sur-Cure (Baryshnikov & David, 2001) confirms this. In Eirós a slight predominance of females is observed (Lopez-Gonzales & Grandal d'Anglade, 2001). In our opinion, the mean value of the lower carnassial length is strongly influenced by the sex ratio of the assemblage, although other factors, mentioned above, may play a role as well.

According to RABEDER (2001), the mean dimensions of cheek teeth of cave bears are not influenced by the sex ratio, but by geological age and phylogenetic position of the assemblage. He further considers that the distribution of the dimensions corresponds to a unimodal Gauss curve as shown in his figure 6 of the greatest crown length of the m1 from the Ramesch-Knochenhöhle; the mean of this assemblage is 29.26 mm. However, according to GODFREY et al. (1993), a mixture of male and female subsamples does not show a bimodality if the means of the males and females are separated by less than two subsample standard deviations, for subpopulation standard deviaton ratios of between 0.4 and 2.5, even if the mixing proportions of the males in the sample fluctuates between 20-80%. The distribution becomes progressively bimodal as the separation of the means increases. In the samples of brown bears from European Russia and Kamchatka the difference between the male and female means is less than two male and female standard deviations (Table 2). Only the brown bear sample from Tibet could indicate a trend to bimodal distribution as the difference between the male and female mean is larger (2.27 mm) than two male or female standard deviations.

It is possible that also in cave bear populations the sexual dimorphism of the carnassials fluctuated. In the Goyet sample, the separation between the female mean and the male mean (2.63 mm) is larger than two male standard deviations or female standard deviations (Table 2). In this sample and in our brown bear samples the male / female standard deviation ratio is larger than 0.4 and smaller than 2.5 (Table 2). RABEDER (2001) does not give the male or female mean of the Ramesch-Knöchenhöhle m1 mixture. However, it is possible that the lower carnassial of the small-sized Alpine cave bears was less sexually dimorphic than that of the large-sized cave bears from Goyet or Mixnitz. Also according to GRANDAL D'ANGLADE (2001), the different degree of sexual dimorphism in different cave bear populations is well marked, especially in the lower jaw.

MORPHOMETRICAL VARIABILITY

In the assemblages from Goyet and Odessa, the mean lengths of the P4, M2, p4, m1 and m3 are larger than from the other assemblages, with the exception of the lower carnassial from Eiros (Tables 3, 4, 6, 7, 9). Eiros has the smallest mean length for the M1 and Medvezhiya for the m2 (Tables 5, 8).The posterior jugal teeth can be grouped according to the geographical position of the assemblages. The greatest average length of the M2 is found in the Odessa assemblage, the largest tooth size occurs in Goyet. The smallest average tooth length occurs at Medvezhiya Cave. In Goyet, Niedzwiedzia and Medvezhiya, the metacone of this tooth is larger than in the other sites (Table 6). The mean metacone index (Lme/GL*100) for the latter sites is larger than 25 (GB4 : 26.1, Ni : 28.0, Me : 25.3), this index from the other sites is smaller than 25 (Od : 24.6, AsC : 22.9, CE : 22.7). According to KURTÉN (1958), juveniles from Odessa with a large paracone had a higher mortality rate than those with a small paracone, due to a less-well-functioning occlusion. It is not clear from our studies if the posterior metacone posed a comparable problem. The cluster analysis in Figure 1, based on the greatest length, length of paracone, length of metacone, greatest width and width across hypocone, shows an interesting subdivision into two groups. The first group unites Goyet, Niedzwiedzia and Medvezhiya, which are situated near the northern border of the distributional range of the cave bear. The second group is constituted by the southern localities (Eiros, Arcy-sur-Cure, Odessa). The differences between these groups are rather small (squared Mahalanobis distances less than 6.1).

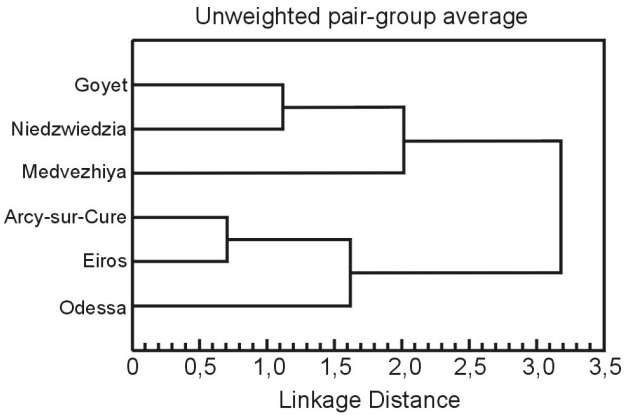

Fig. 1. – Hierarchical tree plot for M2 of *Ursus spelaeus* according to squared Malalanobis distances

The m3 from Goyet are the longest in average. The third molars from Medvezhiya Cave are extremely small. The assemblages of Goyet, Niedzwiedzia and Medvezhiya are characterized by a long talonid (> 14 mm, Table 9). The talonid index (LTa/GL*100) amounts to 51.5 for Goyet, to 52.8 for Niedzwiedzia and reaches the extreme value of 63.8 for Medvezhiya. This index remains below 50 for the other sites (Od : 47.1, AsC : 45.9, CE : 45.8). The cluster analysis given in Figure 2 is based on four measurements (greatest length, length of talonid, greatest width and width of talonid). The analysis subdivides the sites into two groups. The first group includes Medvezhiya Cave, being well-distanced from the second one (squared Mahalanobis distances from 9.20 to 25.17). Within the second group, there are two clusters, one involving more northern sites (Goyet, Niedzwiedzia) and the other uniting the sites located in the south of the distributional range for *U. spelaeus* (Eirós, Arcy-sur-Cure, Odessa).-

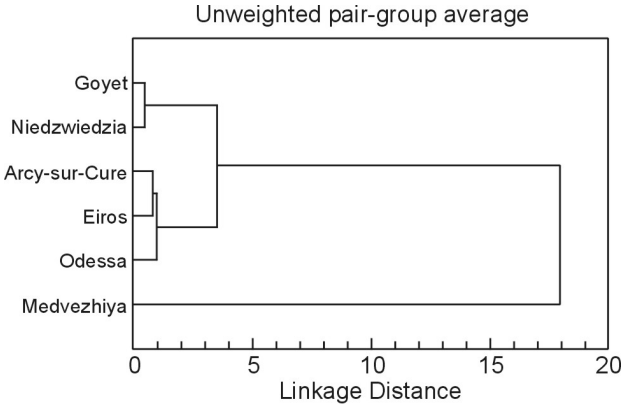

Fig. 2. – Hierarchical tree plot for m3 of *Ursus spelaeus* according to squared Malalanobis distances

TABLE 10

Condylobasal skull length and greatest crown length of the lower carnassial of male Recent brown bears

males	n	CBL	n	GL m1
Ursus a. arctos	24	332.21	20	23.44
Ursus a. piscator	52	370.88	15	25.89
Ursus a. pruinosus	21	340.37	13	26.48

DISCUSSION

The study shows that sexual dimorphism of the canines and of the lower carnassial of the cave bear is as much or more expressed than the dimorphism of these teeth in the Recent brown bear. The morphometry of the cheek teeth of *U. spelaeus* from the studied localities is rather similar. The largest average tooth size occurs in the samples from Odessa (p4, M1, M2) and Goyet (P4, m3). The teeth with the smallest average size were observed in the Medvezhiya Cave (M2, m1, m2, m3). The smaller dimensions may be associated with the dominance of female remains. The cave bears from North Urals were not dwarfed because several teeth found in the Medvezhiya Cave exhibit the maximum length for all the material examined. The lack of important morphological differences could indicate that an exchange of genetic material between adjacent cave bear populations took place. Ancient mitochondrial DNA analysis for the cave bear sometimes reveals difference between individual bears from closely-located caves (Hofreiter et al., 2002). These data may be in contrast with our evidence. However, the differences in mtDNA sequences are passed on via females. The analysis with use of nuclear microsatellite markers produced for modern Alaskan brown bears of insular populations has demonstrated that bears of the ABC Islands, which have previously been shown to undergo little or no female-mediated gene flow with mainland populations (Talbot & Shields, 1996), were found not to be genetically distinct from mainland bears (Paetkau et al., 1998). Possibly this is associated with a different dispersal capability of male and female bears, the females being more phylopatric. Also Orlando et al. (2002) found that extensive gene flow seems to have connected European cave bear populations because two haplogroups cover wide geographic areas.

Most cheek teeth do not show characters useful for the creation of a model of geographical variability in *U. spelaeus*. The exceptions are the M2 and m3. These teeth are in contact in occlusion and are especially active in food processing. Based on these molars, the assemblages can be divided into two well-separated geographical groups. The first group includes the localities situated on the northern boundary of the cave bear's distributional range (Goyet, Niedzwiedzia and Medvezhiya Cave). The second group involves more southern localities (Eirós, Arcy-sur-Cure and Odessa). During the evolution of the genus *Ursus* the posterior cheek teeth were strongly modified, particularly in cave bears (*U. deningeri* Reichenau, 1904 - *U. spelaeus* Rosenmüller, 1794), as these teeth are functionally important for the processing of rough plant food (Rabeder et al., 2000). Therefore, the differences observed in the proportions of the M2 and the m3, in the M2 metacone index and in the m3 talonid index are interpreted as adaptive. The diet of cave bears inhabiting the north of their range might have differed from that in bears occupying more southern regions.

A similar tendency in geographical variability of cheek teeth is found in the recent *U. arctos* in Asia. The brown bears from Tibet demonstrate larger teeth than the animals from northern Siberia; bears from southern Siberia and Mongolia are intermediate (Aristov & Baryshnikov, 2001). The southern brown bears have a more herbivorous diet than those from northern Siberia, whose diet includes a larger proportion of meat and fish (Zhiryakov & Grachev, 1993, Chernyavskiy et al., 1993, Chernyavskiy & Krechmar, 2001). Furthermore, the size of the cheek teeth depends not only on the habitat but of the size of the animal as well. In Table 10 the crown length of the m1 from the males of three brown bear populations is compared with the condylobasal length of the skull. The largest m1 values are found both in the enormous bears from Kamchatka and in the moderate-sized bears of Tibet. Furthermore, sexual dimorphism seems to be larger in the latter group (Table 2). Size differences in Recent brown bear do not necessarily mean that the bears are genetically distinct. The difference in body size between the coastal brown bears of Alaska and those from the interior of Alaska, can be explained by ecological (abundant salmon resource) rather than genetic factors. These populations comprise a single subspecies (*Ursus arctos horriblis* (Ord)) (Paetkau et al., 1998).

Cave bears are presumed herbivores, based on dental morphology and isotope signatures (Kurtén, 1976; Bocherens et al., 1997). Probably the northern cave bear populations had to cope with harder plant food, which needed to be chewed longer; they adapted by modifying their posterior jugal molars. The cave bears from the Ural, due to their most northeastern location, show this adaptation in an extreme form. Furthermore, the moderate sexual dimorphism of the lower carnassial of the cave bears from Goyet could be a consequence of feeding on rough plant material, as the larger sex had to eat more abrasive food to sustain its greater mass. Stable isotope analyses of cave bears from distinct geographic regions confirms this difference in plant food. Bocherens et al. (1997) found significant differences between the $\delta^{13}C$ values in cave bears from layer 1A of the Belgian site of Sclayn, dating from the Middle Weichselian, and sites in southern France. During the Last Glacial, Belgium experienced more severe climatic conditions than southern Europe and the more ^{13}C-depleted collagen in cave bear bones from Sclayn is linked by the authors to the influence of the climatic conditions on plant photosynthesis ^{13}C fractionation. According to Fernandez-Mosquera et al. (2001), depletion of $\delta^{13}C$ can also be caused by the high rate of bone renewal during dormancy. The length of the dormancy in cave bears depends not only on the climatic conditions, but furthermore differs between males and females (Germonpre & Sablin, 2001). Thus, isotope signatures could also depend on the sex of the cave bear bones.

For a better view on the geographical variability of *U. spelaeus*, further research is needed, on well-dated cave bear assemblages, concerning the dental morphometry of

sexed teeth as well as the isotope signatures in collagen from sexed skeletal elements.

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TABLE 3

Measurements of the m1 in *Ursus spelaeus*

m1	n	OR	m	SD	CV
Goyet B4					
GL	42	26.1-34.0	30.95	1.84	5.95
LTr	37	16.5-19.3	18.68	1.05	5.62
LE1	25	3.4-6.5	5.65	0.73	12.92
LE2	25	4.9-7.5	6.04	0.60	9.93
WTr	40	10.3-14.1	12.25	1.05	8.57
WTa	41	13.6-17.0	15.09	0.89	5.90
MW	41	9.6-13.6	11.82	0.90	7.61
Odessa					
GL	45	28.0-34.1	31.24	1.53	4.90
LTr	42	10.2-26.0	19.72	2.08	10.55
LE1	40	4.5-7.5	5.54	0.69	12.45
LE2	40	4.2-8.2	5.59	0.83	14.85
WTr	45	10.8-14.4	12.60	0.82	6.51
WTa	45	13.8-17.2	15.40	0.77	5.00
MW	45	10.9-14.1	11.98	0.64	5.34
Arcy-sur-Cure					
GL	25	27.3-32.5	30.70	1.39	4.53
LTr	25	17.2-20.7	19.33	0.90	4.66
LE1	24	3.4-6.3	5.26	0.72	13.69
LE2	24	3.8-7.1	5.72	0.60	10.49
WTr	25	10.7-13.9	12.34	0.75	6.08
WTa	25	13.1-16.4	14.97	0.53	3.54
MW	25	10.4-12.9	11.56	0.53	4.59
Eiros					
GL	25	27.7-34.0	31.32	1.48	4.73
LTr	25	17.3-21.1	19.42	0.94	4.84
LE1	25	4.3-7.0	5.34	0.86	16.10
LE2	25	3.8-7.2	5.36	0.77	14.37
WTr	25	10.4-13.1	11.67	0.69	5.91
WTa	25	13.4-16.0	14.81	0.76	5.13
MW	25	10.4-12.6	11.19	0.55	4.92
Niedzwiedzia					
GL	28	27.2-32.7	30.49	1.35	4.43
LTr	28	15.7-21.1	19.20	1.11	5.78
LE1	38	4.0-7.4	5.66	0.70	12.37
LE2	38	4.2-7.7	5.81	0.79	13.60
WTr	28	9.7-12.7	11.75	0.70	5.96
WTa	38	11.8-16.0	14.32	0.97	6.77
MW	38	9.1-13.0	11.42	0.79	6.92
Medvezhiya					
GL	24	26.9-33.9	30.11	1.68	5.58
LTr	24	17.3-21.5	19.24	1.15	5.98
LE1	24	4.8-9.6	6.04	1.06	17.55
LE2	24	4.0-6.4	5.14	0.63	12.26
WTr	24	10.8-12.4	11.73	0.58	4.94
WTa	24	12.8-16.2	14.36	0.96	6.69
MW	24	9.8-12.0	10.96	0.60	5.47

TABLE 4

Measurements of the P4 in *Ursus spelaeus*

P4	n	OR	m	SD	CV
Goyet B4					
GL	13	19.4-22.8	21.52	0.99	4.60
LPa	13	10.6-13.4	12.58	0.80	6.36
GW	13	14.9-17.4	15.56	0.74	4.76
MLPC	13	14.2-17.8	16.51	1.05	6.36
Odessa					
GL	50	18.2-24.0	21.30	1.49	7.00
LPa	50	10.3-16.4	13.66	1.18	8.64
GW	50	12.8-17.1	14.83	1.07	7.22
MLPC	50	9.9-18.5	14.80	1.64	11.08
Arcy-sur-Cure					
GL	22	18.9-23.3	20.94	1.33	6.35
LPa	22	11.6-15.3	13.47	0.94	6.98
GW	22	12.7-16.9	14.81	1.12	7.56
MLPC	22	13.3-17.3	15.25	1.28	8.39
Eiros					
GL	17	18.1-21.8	20.41	1.20	5.88
LPa	17	10.9-14.2	12.87	0.87	6.76
GW	17	12.5-16.0	14.38	1.10	7.65
MLPC	17	13.8-17.9	15.84	1.29	8.14
Niedzwiedzia					
GL	9	18.8-21.3	19.92	0.75	3.77
LPa	9	11.1-13.4	12.49	0.70	5.60
GW	9	10.5-14.7	13.14	1.25	9.51
MLPC	9	12.0-16.2	14.40	1.32	9.17
Medvezhiya					
GL	8	19.3-26.0	21.00	2.18	10.38
LPa	8	11.6-14.3	13.07	0.84	6.42
GW	8	13.4-15.1	14.10	0.60	4.26
MLPC	8	13.5-16.6	14.97	0.92	6.15

TABLE 5

Measurements of the M1 in *Ursus spelaeus*

M1	n	OR	m	SD	CV
Goyet B4					
GL	25	25.7-31.1	28.57	1.58	5.53
GW	25	18.6-21.8	20.14	0.99	4.92
Lfp	22	12.5-15.4	14.01	0.68	4.85
Lcp	22	13.3-16.5	15.05	0.88	5.85
LPa	21	10.0-12.8	11.43	0.78	6.82
LMe	21	9.5-11.5	10.51	0.65	6.18
Odessa					
GL	53	27.6-33.4	29.82	1.35	4.53
GW	52	18.5-23.6	20.83	0.98	4.70
Lfp	53	13.1-16.0	14.41	0.69	4.79
Lcp	53	13.9-18.0	15.59	0.91	5.84
LPa	53	9.9-12.4	11.08	0.60	5.42
LMe	53	9.2-11.8	10.37	0.50	4.82
Arcy-sur-Cure					
GL	19	24.9-32.0	28.92	1.98	6.85
GW	19	18.3-22.5	20.16	1.36	6.75
Lfp	19	11.5-15.2	13.69	1.00	7.30
Lcp	19	13.1-17.0	15.08	1.29	8.55
LPa	19	8.6-12.0	10.49	0.87	8.29
LMe	18	8.5-11.7	9.97	0.91	9.13
Eiros					
GL	26	26.0-30.8	28.43	1.32	4,464
GW	26	16.9-21.7	19.85	1.23	6.20
Lfp	26	12.0-14.6	13.56	0.69	5.09
Lcp	26	13.1-16.4	14.72	0.86	5.84
LPa	26	9.0-11.4	10.06	0.53	5.27
LMe	26	8.6-10.7	9.58	0.57	5.95
Niedzwiedzia					
GL	20	26.6-32.8	29.38	1.87	6.36
GW	20	17.8-21.2	19.59	1.17	5.97
Lfp	20	12.0-15.4	14.01	0.88	6.28
Lcp	19	13.2-16.6	14.84	1.07	7.21
LPa	16	10.4-12.2	11.27	0.58	5.15
LMe	16	9.4-11.5	10.72	0.62	5.78
Medvezhiya					
GL	22	26.4-32.6	29.31	1.54	5.25
GW	22	12.5-17.5	14.23	0.98	6.89
Lfp	22	12.9-17.7	15.25	1.20	7.87
Lcp	22	9.3-11.4	10.22	0.59	5.77
LPa	22	8.6-10.7	9.85	0.56	5.69
LMe	22	18.2-22.0	20.16	0.98	4.86

TABLE 6

Measurements of the M2 in *Ursus spelaeus*

M2	n	OR	m	SD	CV
Goyet B4					
GL	22	41.2-53.5	45.95	2.89	6.29
LPa	18	12.6-16.4	14.14	1.09	7.71
LMe	17	10.3-14.4	11.98	1.11	9.27
GW	22	21.1-25.1	23.19	1.09	4.70
WHy	22	18.1-23.3	20.60	1.33	6.46
Odessa					
GL	53	36.7-51.9	46.32	2.87	6.20
LPa	53	12.0-15.4	13.55	0.81	5.98
LMe	53	8.7-15.0	11.40	1.46	12.81
GW	52	19.9-27.1	23.73	1.61	5.81
WHy	53	16.0-23.0	20.42	1.53	7.49
Arcy-sur-Cure					
GL	18	38.5-48.7	45.43	2.53	5.57
LPa	18	12.0-14.8	13.24	0.92	6.95
LMe	18	8.9-12.8	10.40	1.12	10.77
GW	18	21.4-25.1	23.12	1.08	4.67
WHy	18	19.3-23.1	21.29	1.08	5.07
Eiros					
GL	25	41.4-47.1	44.71	1.52	3.40
LPa	25	11.0-14.7	12.96	0.92	7.10
LMe	25	7.6-12.1	10.22	1.05	10.27
GW	25	20.3-25.1	22.61	0.92	4.07
WHy	25	17.8-21.6	20.27	0.78	3.85
Niedzwiedzia					
GL	14	40.6-48.2	44.40	2.40	5.41
LPa	17	12.2-16.2	13.98	1.07	7.65
LMe	14	10.6-14.7	12.43	1.21	9.73
GW	17	19.5-26.4	22.91	1.89	8.25
WHy	14	18.3-21.1	19.55	0.94	4.81
Medvezhiya					
GL	21	39.1-52.6	44.10	3.14	7.12
LPa	21	11.3-18.4	13.03	1.48	11.36
LMe	21	10.1-14.4	11.71	1.07	9.14
GW	21	19.9-27.7	22.72	1.52	6.69
WHy	21	18.1-24.5	20.50	1.51	7.37

TABLE 7

p4	n	OR	m	SD	CV
Goyet B4					
GL	30	13.0-19.0	16.01	1.71	10.68
GW	30	8.4-13.7	10.86	1.27	11.69
Odessa					
GL	56	12.9-18.5	16.14	1.27	7.87
GW	56	9.5-13.0	11.24	0.83	7.38
Arcy-sur-Cure					
GL	28	13.9-17.6	15.44	0.95	6.15
GW	28	9.4-12.8	10.74	0.95	8.85
Eiros					
GL	17	11.3-17.8	15.52	1.63	10.5
GW	17	8.9-12.4	10.64	0.92	8.65
Medvezhiya					
GL	4	14.2-17.5	15.58	1.39	8.92
GW	4	9.4-12.5	10.80	1.33	12.31

TABLE 8

Measurements of the m2 in *Ursus spelaeus*

m2	n	OR	m	SD	CV
Goyet B4					
GL	37	26.8-33.7	30.97	1.74	5.62
LaLTr	26	16.4-20.7	18.40	1.10	5.98
LiLTr	25	13.8-18.3	16.95	1.17	6.90
LaLTa	26	11.0-15.5	13.36	1.13	8.46
LiLTa	23	11.5-15.8	14.32	1.20	8.38
WTr	35	16.3-20.3	18.41	1.11	6.03
WTa	36	16.7-21.0	19.01	1.29	6.79
Odessa					
GL	89	27.5-35.7	31.26	1.64	5.25
LaLTr	89	16.4-21.9	18.73	1.28	6.83
LiLTr	89	13.5-19.6	16.44	1.29	7.85
LaLTa	89	9.9-15.2	12.74	1.21	9.50
LiLTa	89	10.8-18.0	13.99	1.54	11.01
WTr	89	16.4-20.9	18.44	1.01	5.48
WTa	89	17.1-22.1	19.30	1.09	5.65
Arcy-sur-Cure					
GL	23	28.1-32.2	31.51	1.76	5.59
LaLTr	23	17.3-20.6	18.61	0.90	4.84
LiLTr	23	14.7-19.2	16.57	1.13	6.82
LaLTa	23	9.6-13.9	11.84	1.04	8.78
LiLTa	23	10.1-15.3	12.69	1.27	10.01
WTr	23	16.4-21.0	18.23	1.16	6.36
WTa	23	16.0-21.4	18.94	1.31	6.92
Eiros					
GL	24	28.3-34.5	31.36	1.42	4.53
LaLTr	24	15.7-19.9	17.56	1.18	6.72
LiLTr	24	14.4-17.7	15.77	0.84	5.33
LaLTa	24	10.3-14.3	12.48	0.94	7.53
LiLTa	24	11.4-16.0	13.33	1.18	8.85
WTr	24	15.5-20.5	17.66	1.04	5.89
WTa	24	17.1-22.8	19.12	1.27	6.64
Niedzwiedzia					
GL	27	28.0-34.4	31.18	1.98	6.35
LaLTr	27	16.5-20.8	18.54	1.24	6.69
LiLTr	27	13.7-19.7	16.71	1.54	9.22
LaLTa	27	10.6-15.7	12.70	1.08	8.50
LiLTa	27	10.9-16.3	13.65	1.33	9.74
WTr	27	15.6-20.3	18.01	1.24	6.89
WTa	27	16.3-20.9	18.57	1.30	7.00
Medvezhiya					
GL	15	27.4-31.9	29.17	1.35	4.97
LaLTr	15	15.3-18.9	17.02	0.88	5.17
LiLTr	15	14.5-17.2	15.73	0.79	5.02
LaLTa	15	10.0-14.2	11.76	1.26	10.71
LiLTa	15	9.7-15.7	12.60	1.44	11.43
WTr	15	15.5-18.6	17.04	0.89	5.22
WTa	15	15.6-19.9	17.49	1.32	7.55

TABLE 9

Measurements of the m3 in *Ursus spelaeus*

m3	n	OR	m	SD	CV
Goyet B4					
GL	29	24.9-32.4	28.70	2.12	7.39
LTa	15	11.9-16.2	14.77	1.50	10.16
GW	26	17.2-22.1	19.87	1.27	6.39
WTa	27	15.6-21.2	18.66	1.57	8.41
Odessa					
GL	100	21.0-31.4	27.33	2.10	7.68
LTa	100	8.1-16.7	12.86	1.45	11.28
GW	99	17.5-22.7	19.91	1.24	6.23
WTa	99	14.1-22.0	18.79	1.48	7.88
Arcy-sur-Cure					
GL	21	23.8-29.5	27.13	1.47	5.42
LTa	21	10.0-14.7	12.44	1.25	10.05
GW	21	17.1-20.8	19.04	0.96	5.04
WTa	21	15.6-20.6	18.22	1.38	7.57
Eiros					
GL	12	25.2-30.1	27.20	1.78	6.54
LTa	12	10.6-14.6	12.45	1.40	11.24
GW	12	17.4-21.0	19.32	1.17	6.06
WTa	12	14.8-20.3	17.29	1.65	9.54
Niedzwiedzia					
GL	20	24.1-31.0	27.28	1.94	7.11
LTa	20	11.5-17.1	14.40	1.68	11.67
GW	20	17.5-21.2	19.32	1.05	5.43
WTa	19	16.8-20.5	18.23	1.08	5.92
Medvezhiya					
GL	21	21.5-29.7	25.31	2.17	8.57
LTa	21	10.4-20.2	16.15	1.92	11.89
GW	21	15.7-20.5	18.46	1.30	7.04
WTa	21	13.3-19.2	16.74	1.62	9.68

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Study on *Leptoconchus* species (Gastropoda, Coralliophilidae) infesting Fungiidae (Anthozoa : Scleractinia). 1. Presence of nine Operational Taxonomic Units (OTUs) based on anatomical and ecological characters

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ABSTRACT. This paper deals with the *Leptoconchus* infesting Fungiidae. A dichotomous key based on five characters, and sustained by a multivariate analysis (canonical discriminant analysis) based on 11 characters allows clear separation into nine *Leptoconchus* Operational Taxonomic Units (OTUs). The nine OTUs are provisionally labelled OTU1, OTU2,…, OTU9. A single *Leptoconchus* OTU infests several fungiid species whereas a single fungiid species is never infested by different *Leptoconchus* OTUs. Some *Leptoconchus* OTUs infest closely related coral species, whereas other infest distantly related species.

KEY WORDS : *Leptoconchus*, Fungiidae, taxonomy, coral host specificity.

INTRODUCTION

Most species belonging to the genus *Leptoconchus* Rüppell, 1835 have whitish shells, without characteristic sculptures such as spines, nodules, cords, ribs, costae or columellar folds. Adults secrete deposits that cover their protoconch. Despite the lack of characteristic shell sculptures, Sowerby 2nd (1830), Deshayes (1863), and Sowerby 2nd (in Reeve, 1872) distinguished several species of *Leptoconchus* (see Massin, 1982) based mainly on shell shape (globose, lenticular, turbinate, etc). The lack of shell characteristics to separate different species led Sowerby 3rd (1919) to consider that nearly all *Leptoconchus* species represent juveniles of *Magilus* and that the genus *Magilus* Montfort, 1810 includes only one species, i.e., *Magilus antiquus* Montfort, 1810. This view was not shared by subsequent authors (Gohar & Soliman, 1963; Shikama, 1963; Massin, 1982, 1983, 1990; Kosuge & Suzuki, 1985; Zibrowius & Arnaud, 1995) who have since recognised different species based on shell shape, shell aperture, shell surface (smooth or not), presence/absence of an operculum, and coral host.

Shells of *Leptoconchus* species are said to be highly variable depending on the coral they are infesting. However, several ecological studies have shown that these molluscs are host-specific (Robertson, 1970; Massin, 1989, 1990, 2000) and that the coral host had little (Massin, 1990 : Faviidae) or no influence (Massin, 2000 : Fungiidae) on shell morphology.

According to Bouillon et al. (1983) Fungiidae are infested by a single species of *Leptoconchus*. Zibrowius & Arnaud (1995) mentioned also one species of *Leptoconchus* (*L. striatus* Rüppell, 1835) in Fungiidae from the Seychelles. Several other papers (Hoeksema, 1993a, b; Hoeksema & Achituv, 1993) mention the presence of *Leptoconchus* snails in Fungiidae but without referring to (a) given species.

In contrast, evidence from Massin (2000), including burrow observations (location within the coral, opening on oral/aboral side of Fungiidae), the number of adjoining *Leptoconchus* individuals in a coral (pair/cluster) and the deformation of the coral skeleton, suggests that several species of *Leptoconchus* infest Fungiidae. In a first step we will separate the *Leptoconchus* specimens studied into several Operational Taxonomic Units (OTUs) based on anatomical and ecological characters. Then we will try to see, among the Fungiidae, if a single *Leptoconchus* OTU infests more than one coral species, and if the hypothesis holds that a coral species is never infested by two or more *Leptoconchus* OTUs. These phenomena have already been observed for *Leptoconchus* species associated with Faviidae (Massin, 1983).

MATERIAL AND METHODS

Several discriminant characters were selected. Most of these characters were studied only for females. Males were rarely taken into account because the selected characters show few, if any, variations.

Selected characters :

1. Opening of the *Leptoconchus* burrow on coral surface. *Oral surface : 0; aboral surface : 1.* Depending on the *Leptoconchus* species, the opening of the burrow is located on the oral or aboral surface of the fungiid. This character is highly discriminant and corresponds to a different ecology. *Leptoconchus* species with the opening of the burrow on the oral surface of the coral are unable to

achieve lateral movements, whereas those with the opening on the aboral surface can achieve lateral movements only for a short distance after settlement.

2. Female shell height (H) in mm.
3. H/W : ratio of shell height (H) and shell width (W). This character has been used for females (multivariate analysis) and for males and females (dichotomous key).
4. Operculum. *Operculum absent : 0; operculum present : 1*. If present, the operculum is usually reduced, translucent and difficult to observe. It may be easily overlooked or lost; when very reduced, it is no longer firmly attached to the foot.
5. Ho/H : ratio of operculum height (Ho) and shell height (H). This character has been used for females (multivariate analysis) and for males and females (dichotomous key). Ho often represents less than 20% of H.
6. Foot secretion. A*bsent : 0; weak but present : 1*; *well developed : 2*. The burrow is not lined with calcareous secretions from the molluscs except at the level of the foot where an oval calcareous plate is variously developed, and at the level of the short funnel, which connects the burrow with the outside. However, this character has not been taken into account because it is always present.
7. Deformation of coral skeleton. *No deformation : 0; weak deformation : 1; strong deformation : 2*. Several infested corals show no deformation of the coral skeleton (some *Fungia (Danafungia) scruposa* Klunzinger, 1879 and *F. (Lobactis) scutaria* Lamarck, 1801) on either the oral or aboral surfaces. The presence of *Leptoconchus* snails can also induce a week deformation of the septae and/or the costae (*F. (F.) fungites* (Linnaeus, 1758), *F. (Verrillofungia) concinna* Verrill, 1864), or a strong deformation, sometimes with the presence of a prominent bump (*Halomitra pileus* (Linnaeus, 1758), some *F. (D.) scruposa*).
8. Position of the *Leptoconchus* in the coral. DB/R x 100; Ratio between the distance to the coral mouth (DB) and the radius of the coral (R). For round corals, (monostomatous and polystomatous), DB is the distance from the centre of the coral to the burrow opening and R is the radius of the coral. For elongate (oval) corals (monostomatous with a very long mouth slit or polystomatous), DB is the shortest distance (perpendicular) from the opening of the burrow to the long axis of the oval, and R is the half of the short axis of the oval.
9. Number of *Leptoconchus* individuals in a coral. *Leptoconchus specimens alone : 0; Leptoconchus specimens in pair : 1; Leptoconchus specimens in cluster (three and more) : 2*. Generally, there are at least two *Leptoconchus specimens* together (male and female) to ensure reproduction (there is an internal fecundation). In some species, isolated female specimens have been observed over long time periods. Clusters (up to 15 specimens) have also been observed (*Halomitra pileus* (Linnaeus, 1758), *Heliofungia actiniformis* (Quoy & Gaimard, 1833)). If one coral harbours several pairs (two-three) of *Leptoconchus* individuals it will be noted as "1".
10. Shell top. *Shell top flat : 0 : shell top with a visible spire but reduced : 1; shell top with a prominent spire : 2.* Most of the female *Leptoconchus* individuals living in Fungiidae have a flat shell top, although some (living in thick corals or living in the prominent bumps of a coral) present a more or less well-developed spire.
11. Rostrum. *Rostrum absent : 0; rostrum present : 1*. The rostrum (attenuated extremity of last whorl) is sometimes prominent as for the *Leptoconchus* specimens infesting *Fungia (Danafungia) scruposa* or *Halomitra pileus* corals. However, most of the time it is reduced or absent. The *Leptoconchus* specimens infesting *F. (F.) fungites* corals are highly variable regarding the presence/absence of a rostrum.

Three hundred seventy eight female snails infesting 303 corals, coming from Papua New Guinea and the Maldives, have been observed and included in the matrix. The following fungiid species are included in the present work :

Fungia (Cycloseris) costulata Ortmann, 1889, *F. (C.) fragilis* (Alcock, 1893), *F. (C.) tenuis* Dana, 1846, *F. (Wellsofungia) granulosa* Klunzinger, 1879, *F. (Pleuractis) gravis* Nemenzo, 1955, *F. (P.) paumotensis* Stutchbury, 1833, *F. (Verrillofungia) concinna* Verrill, 1864, *F. (V.) repanda* Dana, 1846, *F. (V.) spinifer* Claereboudt & Hoeksema, 1987, *F. (Lobactis) scutaria* Lamarck, 1801, *Herpolitha limax* (Esper, 1797), *Polyphyllia talpina* (Lamarck, 1801), *P. novaehiberniae* (Lesson, 1831), *Heliofungia actiniformis* (Quoy & Gaimard, 1833), *F. (Danafungia) horrida* Dana, 1846, *F. (D.) fralinae* Nemenzo, 1955, *F. (D.) scruposa* Klunzinger, 1879, *F. (F.) fungites* (Linnaeus, 1758), *Halomitra pileus* (Linnaeus, 1758), *Ctenactis albitentaculata* Hoeksema, 1990, *C. crassa* (Dana, 1846), *C. echinata* (Pallas, 1766), *Sandalolitha dentata* Quelch, 1884, *S. robusta* (Quelch, 1886), *Zoopilus echinatus* Dana, 1846, and *Podabacia crustacea* (Pallas, 1766). Relationships between these corals are based on the cladogram of Hoeksema (1989 : fig. 673).

Leptoconchus snails known only from one to two females (e.g. in *Fungia (Pleuractis) seychellensis* Hoeksema, 1993 from the Seychelles; Hoeksema, 1993b; in *Lithophyllon undulatum* Rehberg, 1892 from Indonesia; Massin 2000) or from traces (burrow openings) in a single coral (*Cantharellus jebbi* Hoeksema, 1993 from Papua New Guinea; Hoeksema, 1993a) were not taken into account in the present work.

The dichotomous key is based on the study of more than 1000 *Leptoconchus* specimens (males and females) coming from 430 corals collected in Papua New Guinea, Indonesia and the Maldives. Corals from the Maldives included basically only *Fungia (Fungia) fungites*. They have been summed with the *F. (F.) fungites* from Papua New Guinea because for characters 3 and 5, statistically, they did not show differences (see table 1).

Characters 1, 3, 4, 5 and 9 have been taken into account for the dichotomous key. The coral host is only used as an added value and not as a discriminant character.

This key was tested using multivariate analysis. Simple linear regression models were used to test that there was no correlation between the 11 variables. Tests were also used to check that the data represent a random sample from a normal distribution using the Shapiro & Wilk (1965) statistic. A canonical discriminant analysis was used to separate the different Operational Taxonomic Units (OTUs), a classification variable, using characters 1

TABLE 1

Measures of characters 3 and 5 for the *Leptoconchus* specimens infesting *Fungia (Fungia) fungites* (LINNAEUS, 1758) from the Maldives and PNG. F.(F.)f. = *F. (F.) fungites*; H : shell height; Ho : operculum height; PNG = Papua New Guinea; W : shell width.

Character	F.(F.)f. Maldives	F.(F.)f. PNG	F.(F.)f. Maldives + PNG
3 (H/W shell ♀)	0.79 ± 0.09 (n = 143)	0.84 ± 0.11 (n= 48)	0.80 ± 0.10 (n= 191)
3 (H/W shell ♂)	1.37 ± 0.20 (n= 131)	1.32 ± 0.16(n= 36)	1.36 ± 0.20 (n= 167)
5 (Ho/H ♀ X 100)	8.58 ± 2.71 (n= 120)	7.64 ± 2.05 (n= 35)	8.40 ± 2.60 (n= 155)

to 11 as quantitative variables (KSHIRSAGAR, 1972). Mahalanobi's distances were computed and species were hierarchically clustered using UPGMA method. All analyses were performed using SAS/STAT® software's capabilities (SAS INSTITUTE INC., 1990).

RESULTS

Taking into account the most discriminant characters observed, nine *Leptoconchus* OTUs can be distinguished. They will be provisionally numbered as OTU1, OTU2,…, OTU9.

The dichotomous key allows separation of the nine OTUs. Some of them are very closely related to each other (e.g. OTU8 and OTU9, and OTU5 and OTU6). The most discriminant characters are 1, 3 and 4. Except for OTU7, *Leptoconchus* individuals are infesting either polystomatous or monostomatous corals but not both (Fig. 1).

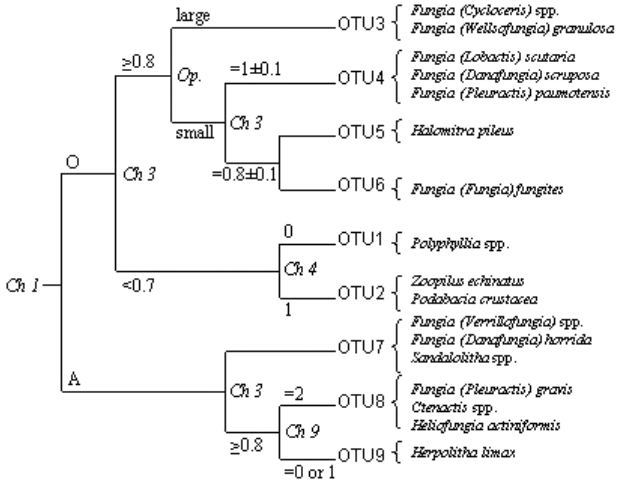

Fig. 1. – Key of the nine *Leptoconchus* species associated with Fungiidae. Ch: character; op: operculum.

Character one allows separation into two groups of *Leptoconchus* : group 1 (OTUs1-6) versus group 2 (OTUs7-9).

In group 1 (with an oral opening of the siphon), OTUs1-6 can be distinguished by characters 3, 4, 5 and 9. Once again, the first discriminant character used is character 3, which separates two groups of *Leptoconchus* : OTU1 and OTU2 versus OTUs3-6.

The lenticular shells with H/W <0.7 (OTU1 and OTU2) are separated by character 4. OTU1 and OTU2 are also separated by their coral hosts (Fig. 1) : OTU1 infests *Polyphyllia talpina* and *P. novaehiberniae* whereas OTU2 infests *Zoopilus echinatus* and *Podabacia crustacea*.

The globose shells with H/W≥0.8 (OTUs3-6) are close to each other but can be separated by characters 3, 4, 5 and 9. OTU3 is clearly separated from OTUs4-6 using characters 4 and 5. Character 3 separates OTU4 from OTU5 and OTU6, whereas character 9 separates OTU5 from OTU6. However, if we are dealing with an early infestation of OTU5, only one or two mollusc specimens will be present in the coral host and the distinction between OTU5 and OTU6 is no longer possible unless the coral host is known (Fig. 1).

In group 2 (with an aboral opening of the siphon), OTUs7-9 can be distinguished by characters 3, 4, 5 and 9. OTU7 is lenticular, whereas OTU8 and OTU9 are globose. OTU8 and OTU9 are very close to each other, but are separated by character 9 and by the coral host (Fig. 1) : OTU8 is found in *Fungia (Pleuractis) gravis*, *Ctenactis albitentaculata, C. crassa, C. echinites* and *Heliofungia actiniformis* whereas OTU9 is exclusive to *Herpolitha limax.*

The key has been established without taking the coral host into account. If coral hosts are listed for each *Leptoconchus* OTU (Fig. 1), it must be noted that a *Leptoconchus* OTU can infest several coral species but that a coral species is never infested by different *Leptoconchus* OTUs. Moreover, a single *Leptoconchus* OTU can infest closely related corals or distantly related corals (see Table 2). For Fungiidae relationships see HOEKSEMA, 1989.

TABLE 2

Operational Taxonomic Units (OTUs) associated with closely related and distantly related corals

	OTU	Coral species
OTU infesting closely related corals	2	*Zoopilus echinatus*, *Podabacia* spp.
	3	*F. (Cycloseris)* spp., *F. (Wellsofungia) granulosa*
	5-6	*Halomitra pileus, F. (Fungia) fungites*
OTU infesting distantly related corals	4	*F. (Danafungia) scruposa, F. (Lobactis) scutaria, F. (Pleuractis) paumotensis*
	7	*F. (Verrillofungia)* spp., *F. (Danafungia) horrida, Sandalolitha* spp.
	8	*F. (Pleuractis) gravis*, *Ctenactis* spp., *Heliofungia actiniformis*

Three canonical discriminant analyses were performed. Using all the eleven characters, the nine OTUs are significantly separated into two clusters ($p<0.01$) : group 1 (OTUs1-6) and group 2 (OTUs7-9). The discrimination between these clusters is based on the opening of the burrow on the fungiid surface (character 1). All characters except 1, 4 and 5 were then used to perform further canonical discriminant analyses on each group. Characters 4 and 5 were removed due to the few data available (Table 3). For group 1, 5 new clusters were significantly discriminated ($p<0.01$) on the basis of characters 3 and 7 (Fig. 2). The character 3 aimed to significantly separate 3 clusters in group 2 (Fig. 3). Fig. 4 presents the UPGMA tree inferred from Mahalanobi's distances computed at the three levels of the analysis. This tree presents a structure similar to the dichotomous key (Fig. 1). Differences were observed in the relations between the OTUs4-6 and for the positions of OTU1 and OTU2.

TABLE 3

Quantity of data available for the multivariate analysis.
OTU : Operational Taxonomic Unit

OTU	N (all data)	N (without Ch4 & Ch5)
1	1	10
2	1	1
3	6	20
4	4	43
5	5	16
6	30	73
7	53	60
8	30	39
9	11	11
Total	140	273

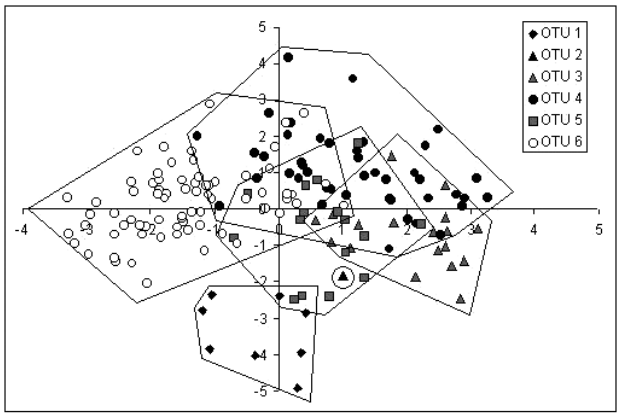

Fig. 2. – Canonical discriminant analysis of the OTUs 1 to 6 using characters 2 to 4 and 7 to 11. (can1=-0.25*ch2+3.60*ch3-0.89*ch6+1.53*ch7-0.02*ch8+0.73*ch9+0.35*ch10-0.32*ch11; can2=0.23*ch2+6.21*ch3+0.43*ch6-0.43*ch7-0.01*ch8-0.18*ch9-0.39*ch10-0.09*ch11)

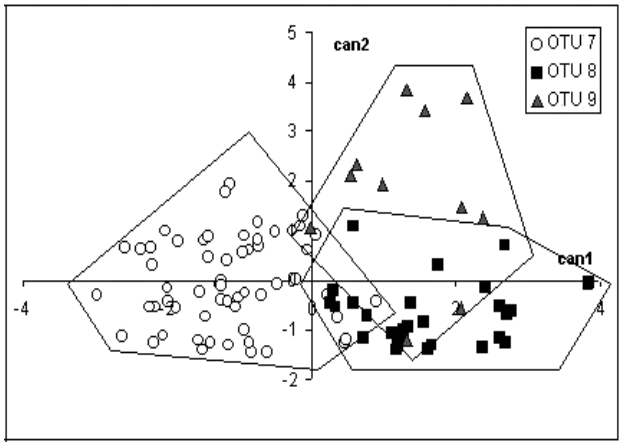

Fig. 3. – Canonical discriminant analysis of the OTUs 7 to 9 using characters 2 to 4 and 7 to 11. (can1=0.29*ch2+5.74*ch3-0.43*ch6-0.44*ch7-0.02*ch8+0.58*ch9-0.24*ch10-0.001*ch11; can2=-0.02*ch2+3.20*ch3+0.52*ch6+1.16*ch7-0.002*ch8-0.74*ch9*0.14*ch10+1.56*ch11)

DISCUSSION

Character 1 is the most discriminant, and allows separation of OTUs1-6 (group 1) from OTUs7-9 (group 2), no matter which analysis is used.

Some small discrepancies appear in group 1 between the key and the UPGMA tree. In the key OTU1 and OTU2 are closely related but clearly separated from each other by the presence/absence of an operculum. In the UPGMA tree OTU2 appears as an outgroup versus OTU1 and OTUs3-6. This is most probably due to the fact that very few data are available for OTU2 and particularly for character 4 (presence/absence of an operculum). Consequently, character 4 has not been taken into account in the UPGMA tree.

In the key, OTU5 and OTU6 are closely related and only the presence of a cluster of molluscs versus presence of a pair of molluscs allows their separation. In the UPGMA tree, using character 7 (deformation of coral skeleton) OTU5 and OTU6 are clearly separated, OTU5 being even closer to OTU4 than to OTU6.

In group 2, canonical discriminant analysis and the UPGMA tree provide the key ordination, separating OTU8 and OTU9.

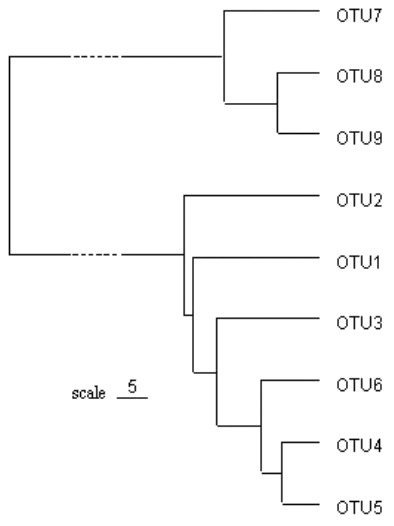

Fig. 4. – UPGMA tree inferred from Mahalanobi's distances computed at the three levels of the analysis.

The dichotomous key has been established using a maximum of characters linked to the molluscan anatomy (characters 3, 4, and 5). The two other anatomical characters (10 and 11) are highly variable and not very discriminant (see canonical analysis of group 1). The emphasis on anatomical characters is dictated by the fact that ecological characters are seldom available from museum collections. For the usefulness of the key it is better to take into account a maximum of anatomical characters. Nevertheless, many ecological data are very discriminant (mainly characters 1, 7, and 9) and allow separation of OTUs in the key as well as in the canonical discriminant analysis and the UPGMA tree.

The nine *Leptoconchus* OTUs selected here present enough discriminant characters to be easily separated from each other. Their taxonomic status will be discussed in a forthcoming paper.

The study of the *Leptoconchus* specimens infesting Fungiidae has shown that a single *Leptoconchus* OTU can infest several corals, whereas a single coral is never infested by several *Leptoconchus* OTUs. This is very similar to the observations done on *Leptoconchus* specimens infesting Faviidae (Massin 1983). Other molluscs infesting living colonies of Scleractinia as *Lithophaga* species (Kleeman, 1980; Morton, 1983) are less selective in their coral/host relationships. For these bivalves, several species can be present in a single coral host. Different *Epitonium* species associated with Fungiidae are also able to infest a single coral host : e.g. *Epitonium costulatum* (Kiener, 1838), *E. ingridae* Gittenberger & Goud, 2000 and *E. twilae* Gittenberger & Goud, 2000 are associated with *Herpolitha limax* (see Gittenberger et al., 2000).

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ANNEX

	Key for the *Leptoconchus* OTUs (Operational Taxonomic Units) infesting Fungiidae.	
	- *Leptoconchus* with the burrow opening on the oral side of the coral	1
	- *Leptoconchus* with the burrow opening on the aboral side of the coral	2
1 -	H/W females <0.7	3
-	H/W females ≥0.8	4
3 -	Operculum absent, often in group >2	OTU1
-	Operculum present, alone or in pair	OTU2
4 -	Operculum present, >10% of shell height for females and >14% for males	OTU3
-	Operculum present or absent, if present <10% of shell height (males & females)	5
5 -	H/W females 1.00 ± 0.10	OTU4
-	H/W females 0.80 ± 0.10	6
6 -	H/W males 1.13 ± 0.18, 50% of the examined specimens living in groups >3	OTU5
-	H/W males 1.35 ± 0.21, never living in group >3	OTU6
2 -	H/W females 0.70 ± 0.10, H/W males 1.00 ± 0.10, operculum always present, >15% of shell height	OTU7
-	H/W females ≥0.80 ± 0.10, H/W males ≥1.10 ± 0.10, operculum present or absent, if present (less than 20% of the specimens examined) <12% of shell height	7
7 -	H/W females 0.80 ± 0.10, H/W males 1.10 ± 0.10, very often in groups >3	OTU8
-	H/W females 0.90 ± 0.10, H/W males 1.22 ± 0.10, nearly always alone or in pair (90% of the specimens examined)	OTU9

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Microvascular architecture in the central nervous system of *Ambystoma mexicanum* (Caudata, Ambystomatidae). A light, and transmission and scanning electron microscopy study

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ABSTRACT. A combination of light, and scanning and transmission electron microscopy of vascular corrosion casts was utilized to study the structure and spatial organization of intraparenchymal microvessels in the central nervous system of the urodele *Ambystoma mexicanum* (Shaw, 1789). The angioarchitectural pattern consists of both single and paired vessels, but the single ones are prevalent. The meningeal vessels give origin to both single and paired elements. The paired vessels are bent and twisted and extend into the neural wall to various depths. Their two limbs are apposed throughout their length and are connected by a U-shaped terminal loop. The cross-sectioned single and paired vessels have variable diameters. The hairpin-shaped vascular pairs do not branch into secondary loops and no anastomotic intraparenchymal connections are observed with single or paired neighbouring structures. All intraparenchymal vessels are structurally interpreted as capillaries. The paired vessels of *Ambystoma mexicanum* resemble those found in a few vertebrates whose brain vascular pattern is represented only by paired structures.

KEY WORDS : Amphibia; central nervous system; vasculature; capillary; corrosion casting.

INTRODUCTION

Each organ has a peculiar angioarchitectural pattern, which is related to the functions of its different tissues and of the whole organ. The structural and spatial aspects of the central nervous system (CNS) vessels have been studied in various vertebrates (Bubis & Luse, 1964; Bundgaard, 1982; Lazzari & Franceschini, 2000), but conventional techniques based on light microscopy have failed to visualize both the general three-dimensional organization and the fine structure of tissue vasculature. Investigations of the spatial disposition of microvessels were difficult until a method based on scanning electron microscopy (SEM) observation of methyl methacrylate plastic casts was devised (Murakami, 1975). The use of a new low-viscosity plastic resin in combination with the high resolving power and the great depth of field of the SEM stimulated studies on the microangioarchitecture in many tissues and organs (see Christofferson & Nilsson, 1990; Lametschwandtner et al., 1990).

To date there have been several studies on the vascular features of anuran brains, mostly using the corrosion casting technique (see Lametschwandtner et al., 1980; Albrecht et al., 1980; Lametschwandtner, 1982 for references), whereas SEM research and ultrastructural studies on the brain vasculature of caudates mainly concern *Triturus carnifex* (Laurenti, 1768) (Ciani & Franceschini, 1984; Franceschini et al., 1988; Lazzari et al., 1991). The only information about the brain vasculature of *Ambystoma mexicanum* (Shaw, 1789) is reported by Roofe (1935) and more recently by Ciani et al. (1989) in a cytochemical investigation. These preliminary studies on *Ambystoma mexicanum* report the presence of a vascular model of the CNS that differs from the two main patterns of CNS vascularization found in vertebrates. These works examine neither the ultrastructure nor the overall three-dimensional organization of these vessels. It appears important to investigate these aspects as they could provide information on the evolution of CNS blood supply.

Therefore, this study was undertaken to elucidate the angioarchitectural pattern and the ultrastructural features of CNS microvessels in *Ambystoma mexicanum* by a combination of light, and transmission and scanning electron microscopy techniques. The results will be useful for comparative analyses of brain vascularization patterns, not only in the amphibians, but also in other vertebrates.

MATERIALS AND METHODS

Animals

Twenty-four metamorphosed *Ambystoma mexicanum* of both sexes (70-85 g body weight, 19-23 cm total length) were obtained from neotenic animal stock raised in our Department. Metamorphosis was induced by Eutirox (Bracco, Milano, Italy) : 2-3 pills were administered over a period of a week inside bits of meat. The urodeles were maintained in terra-aquaria at room temperature in a natural light-dark cycle and fed *ad libitum* with bits of

meat. All procedures were in accordance with the guidelines of the European Communities Council Directive (86/609/CEE), the current Italian legislation for the use and care of animals, and conform to the guidelines of the U.S. National Institute of Health. This study was also approved by the Ethic-Scientific Committee of the University of Bologna. The amphibians were sacrificed by immersion in 0.1% 3-aminobenzoic acid ethyl ester (Sigma Chemical Co., St. Louis, Missouri), and the blood was removed from the vascular system by intracardiac perfusion with 50 ml of Holtfreter's physiological solution containing heparin (3 IU/ml). They were perfusion-fixed with 150 ml of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). All solutions were kept at 4˚C and propelled by a Gilson Minipulse 3 peristaltic pump operating at a constant flow of 5 ml/min.

Light microscopy

The brains and spinal cords of eight urodeles were removed and immersion-fixed in the same fixative used for perfusion for an additional 4 hours at 4˚C. After washing overnight in 0.1 M phosphate buffer (pH 7.4) at 4˚C, they were dehydrated with ethanol and embedded in Paraplast Plus (Sherwood Medical, Athy, Ireland; melting point 55-57˚C). Coronal 10 µm thick sections were mounted on poly-L-lysine (Sigma Chemical Co., St. Louis, Missouri) coated slides and dried. The sections were deparaffinized, hydrated, stained with haematoxylin-eosin solution and then coverslipped with Permount (Fisher Scientific Co., Pittsburgh, Pennsylvania).

Transmission electron microscopy

After washing overnight in 0.1 M phosphate buffer (pH 7.4) at 4˚C, the brains and spinal cords of eight amphibians were coronally-sectioned into 100 µm thick tissue slices using a Vibratome 1000 (Lancer). The slices were then postfixed in 0.1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 hour at 4˚C. After a buffer rinse, tissue slices were dehydrated in acetone and embedded in Durcupan ACM (Fluka Chemical Co., Buchs, Switzerland). Semithin sections, 0.5-1 µm thick, were stained with toluidine blue. Thin sections were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate solutions and examined in a CM 100 Philips electron microscope operating at 80 kV.

Scanning electron microscopy

Eight urodeles, perfusion-fixed as above, were processed for vascular corrosion casting. After their fixation, 60 ml of freshly prepared low-viscosity methyl methacrylate (Gannon, 1981) were injected manually at a constant rate through a cannula inserted in the conus arteriosus at room temperature. After 30 min the injected specimens were placed in a water bath at 60˚C overnight to accelerate and complete the resin polymerization. Afterwards, the heads were immersed in 20% KOH at 60˚C for 24 hours for soft tissue ablation. The solution of KOH was replaced every 6 hours and the endocast washed with a fine stream of water to remove eroded tissue fragments still adhering to the plastic surface. Bone decalcification was then carried out with 2.5% HCl. Finally the casts were treated with 5% aqueous solution of trichloroacetic acid to remove any product of saponification still adhering, and thoroughly rinsed several times in distilled water. The clean replicas were frozen in distilled water at -20˚C and the ice blocks were freeze-dried to prevent vessel collapse (Lametschwandtner et al., 1990). The casts were mounted on aluminium stubs by means of conductive bridges with silver conductive glue, gold-coated in a Bio-Rad SC 502 SEM Coating System and examined in a JEOL JSM-5200 scanning electron microscope at an accelerating voltage of 15 kV.

RESULTS

Light microscopy

All the regions of the *Ambystoma mexicanum* CNS had a parenchymal vascularization with both single and paired vessels (the single ones being prevalent), irregularly arranged throughout the neural wall (Fig. 1A, B). The vascularization arose from the meningeal vessels, from which originated both single and paired elements (Fig. 1C, D). Although the initial track of the microvascular pairs penetrated into the nervous parenchyma almost perpendicularly to the brain surface, they seldom had a rectilinear course in the nervous tissue and the majority of them were twisted and curved (Fig. 1D), which made them appear transversely or obliquely cut in the CNS sections. In each vascular pair the two adjacent limbs were connected by a narrow U-shaped terminal loop (Fig. 1E). The hairpin-shaped vascular pairs extended into the CNS wall to various depths. Some penetrated from the meningeal layer into the superficial nervous tissue for a short distance, others reached deeper layers penetrating into the gray matter and sometimes contacted the ependymal layer.

In tissue sections the two limbs of a pair did not link with the surrounding single or paired vessels. Moreover hairpin-shaped vascular loops did not appear to branch.

Transmission electron microscopy

Both single and paired vessels were present in the nervous tissue (Fig. 2A, B). The paired structures consisted of two adjoining vessels leaning against each other and separated by a thin wall (Fig. 2B). In the various vascular pairs the two adjacent vessels presented the same structure. Both paired and single vessels had similar ultrastructural characteristics. The vascular wall was unfenestrated, and the endothelial cells were thin except in the nuclear region (Fig. 2A, B, C). The nucleus appeared flattened and elongated with chromatin masses placed adjacent to the nuclear envelope. In the paired vessels the nucleus could be located in any part of the 8-shaped vascular outline, and could even enter into the intervascular wall (Fig. 2D). The endothelial lumenal surface had numerous short, finger-like protrusions, which extended in the peripheral zone of the vascular lumen (Fig. 2D, E, F). In the endothelial cells, organelles were mainly located in the region next to the nucleus, where the endothelium thickness increased (Fig. 2C). A few vesicular profiles were free within the endothelial cytoplasm, others were confluent with the endothelial surface membranes appear-

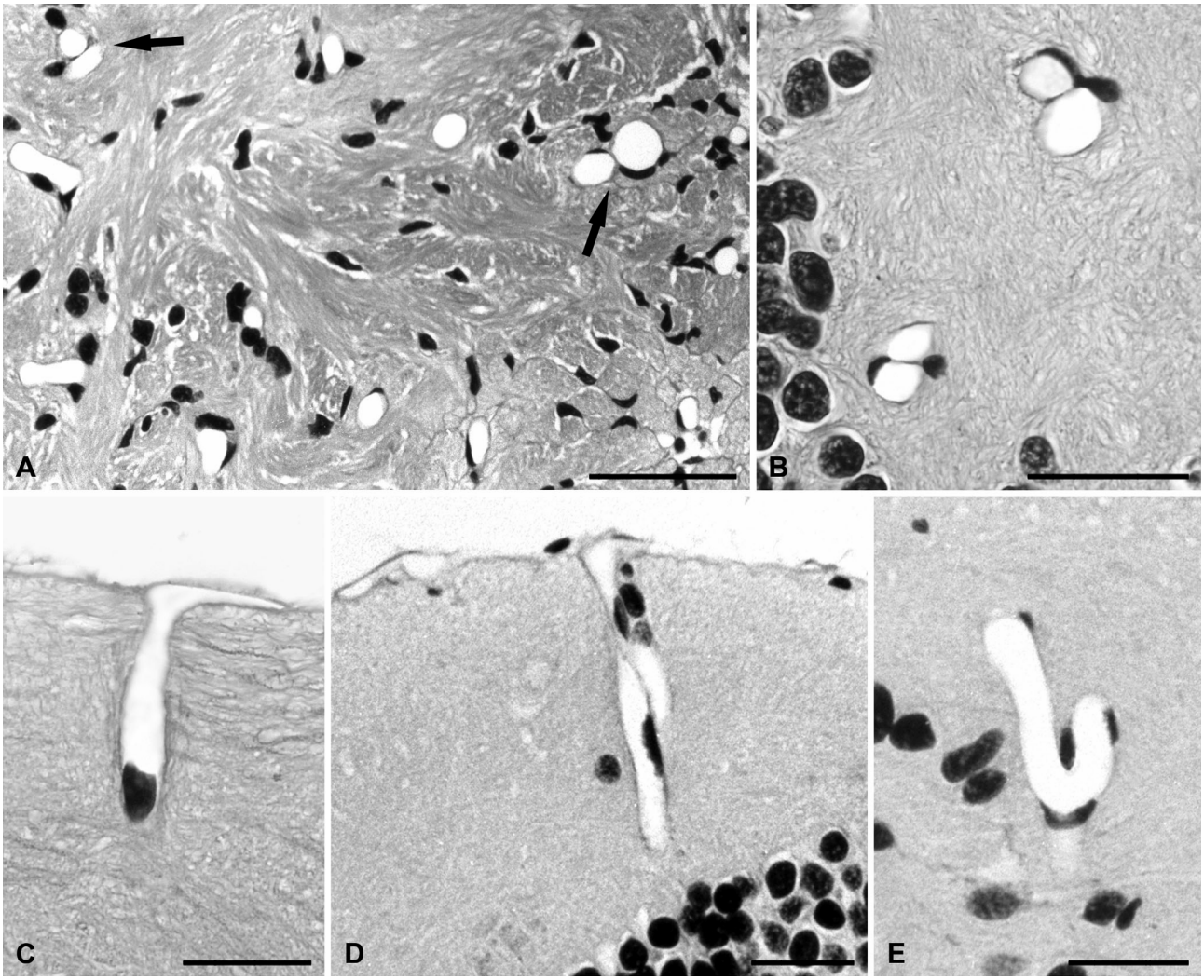

Fig. 1. – Micrographs of *Ambystoma* CNS sections showing parenchymal microvessels. **A** : A few paired vessels (arrows) appear among the single vascular elements in the olfactory bulb. Scale bar, 100 µm. **B** : Two transversely sectioned vascular pairs in the deepest region of the telencephalic white matter. Scale bar, 50 µm. **C** : A single microvessel originates from the meningeal vasculature in the optic tectum. Scale bar, 50 µm. **D** : A pair of tightly apposed, twisted and radially-oriented microvessels originates from the meningeal vasculature in the diencephalon. Scale bar, 50 µm. **E** : A terminal endloop connects the adjacent microvessels of a vascular pair in the medulla oblongata. Scale bar, 50 µm.

ing as pits (Fig. 2E, F). The overlapping endothelial cell edges were connected by tight junctions (Fig. 2C). Neither single nor paired vessels had a smooth muscle cell investment, but sometimes both were associated with pericytes whose processes extended into the endothelial basal membrane. These cells had a nucleus and cytoplasm similar to those found in endothelial cells, but did not constitute a continuous covering. In particular, in the paired vessels, pericytes sometimes appeared to be wedged between the adjacent vascular elements (Fig. 2D, F) with thin processes entering into the thinner part of the intervascular wall (Fig. 2E).

In both single and paired vessels the neuropilar side of the endothelial basal membrane was lined almost continuously by a layer of perivascular glial endfeet that appeared flattened on the basal membrane and were rich in mitochondria (Fig. 2C, D, F). In the paired vessels these perivascular glial cell processes were never found entering into the wall separating adjoining elements; therefore this wall was generally composed of the thin endothelium of each adjacent vessel separated by a single basal membrane. A slight increase in thickness could be due not only to the presence of pericytic cell processes but also to somata in the basal membrane or to endothelial nuclei in one or both endothelial sides.

The same ultrastructural characteristics were found in tissues from both sexes.

Scanning electron microscopy

The features of the vascular pattern were uniform in the different areas of the *Ambystoma mexicanum* CNS in both sexes as revealed by whole vascular casts as well as dissected region moulds. In the vascular casts at the pial surface the meningeal vessels appeared as single elements with a rather winding course (Fig. 3A). They generally forked repeatedly with different angles into two smaller branches, which finally gave rise to the vascular network below (Fig. 3B). The intraparenchymal vessels had a similar branching pattern (Fig. 3C) and sometimes revealed a

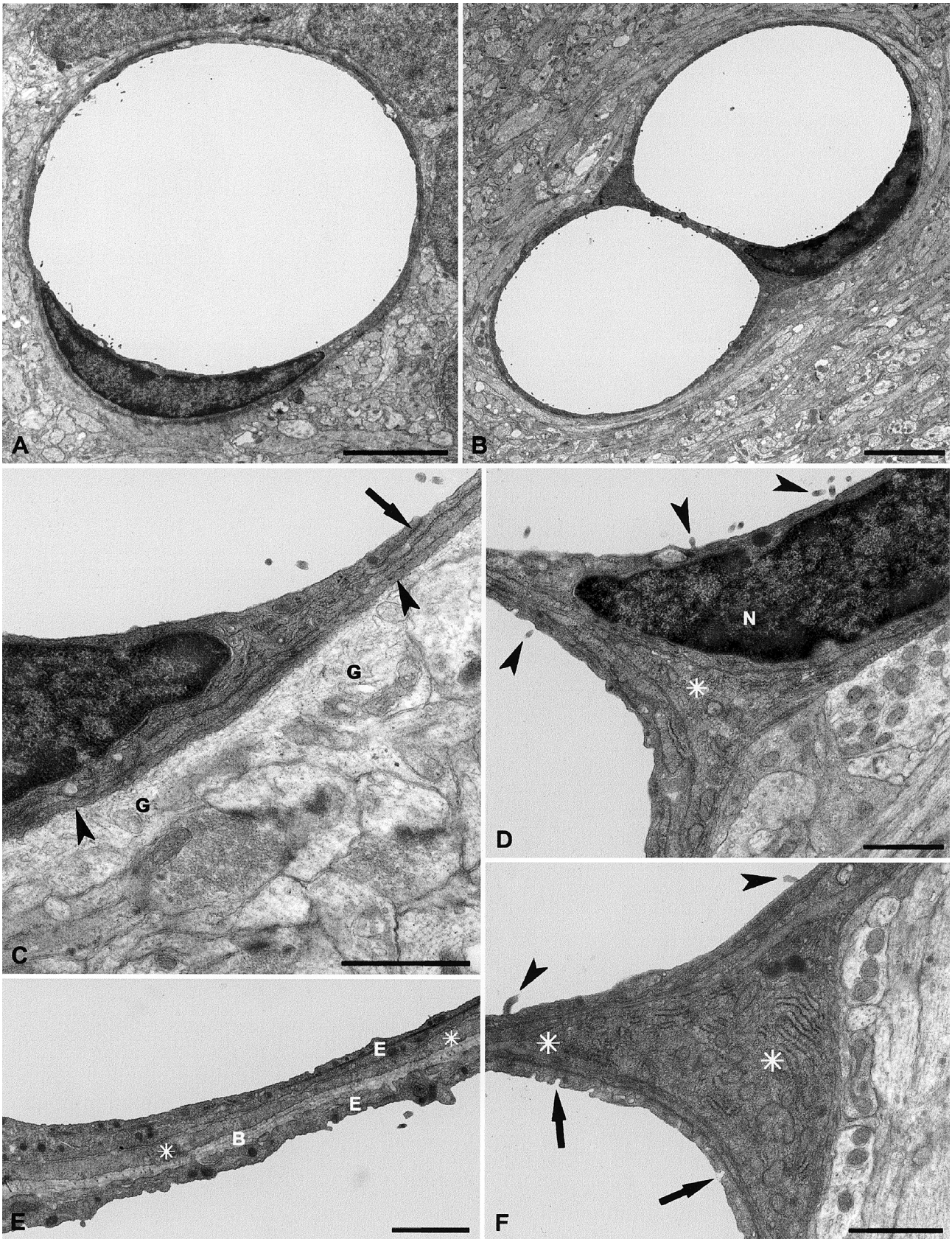

Fig. 2. – Transmission electron micrographs of *Ambystoma* CNS microvessels. **A** : A transversely-sectioned single vessel in the telencephalon showing an elongated and flattened endothelial cell nucleus. Scale bar, 5 µm. **B** : Transverse section of a telencephalic vascular pair with the two closely applied limbs showing a slightly different size. An endothelial cell nucleus is clearly observed in one vascular limb. Scale bar, 5 µm. **C** : Glial endfeet (G) line the endothelial basal membrane (arrowheads) of a mesencephalic single vessel. A tight junction (arrow) links overlapping endothelial cell edges. Scale bar, 1 µm. **D** : An endothelial nucleus (N) penetrates into the thickest tract of the intervascular wall, which includes a pericytic cell process (asterisk). Some finger-like protrusions (arrowheads) arise from the luminal endothelial surface. Scale bar, 1 µm. **E** : A pericytic process (asterisks) enters into the intervascular wall. Scale bar, 1 µm. B, basal membrane; E, endothelial layers. **F** : A pericytic cytoplasmatic mass (asterisks) extends laterally between the two adjacent vessels and penetrates into the intervascular wall. Pits (arrows) and finger like protrusions (arrowheads) appear on the endothelial luminal surface. Scale bar, 1 µm.

winding route with U-shaped loops connected in series (Fig. 3D).

In the vasculature of all CNS regions numerous paired vessels also appeared penetrating from the meningeal surface. They were irregularly spaced in relation to both themselves and the single elements, and were variously curved and inclined with respect to the meningeal surface. In each vascular pair the two elements were apposed all along their routes with a narrow U-shaped loop that connected the two limbs, giving rise to a hairpin-like structure (Fig. 3E, F). Vessels were variously bent and twisted. In the vascular corrosion casts, SEM observations did not show direct anastomoses either between the two closely applied limbs or with adjacent vascular loops and single vessels. The hairpin-shaped vascular pairs were not ramified.

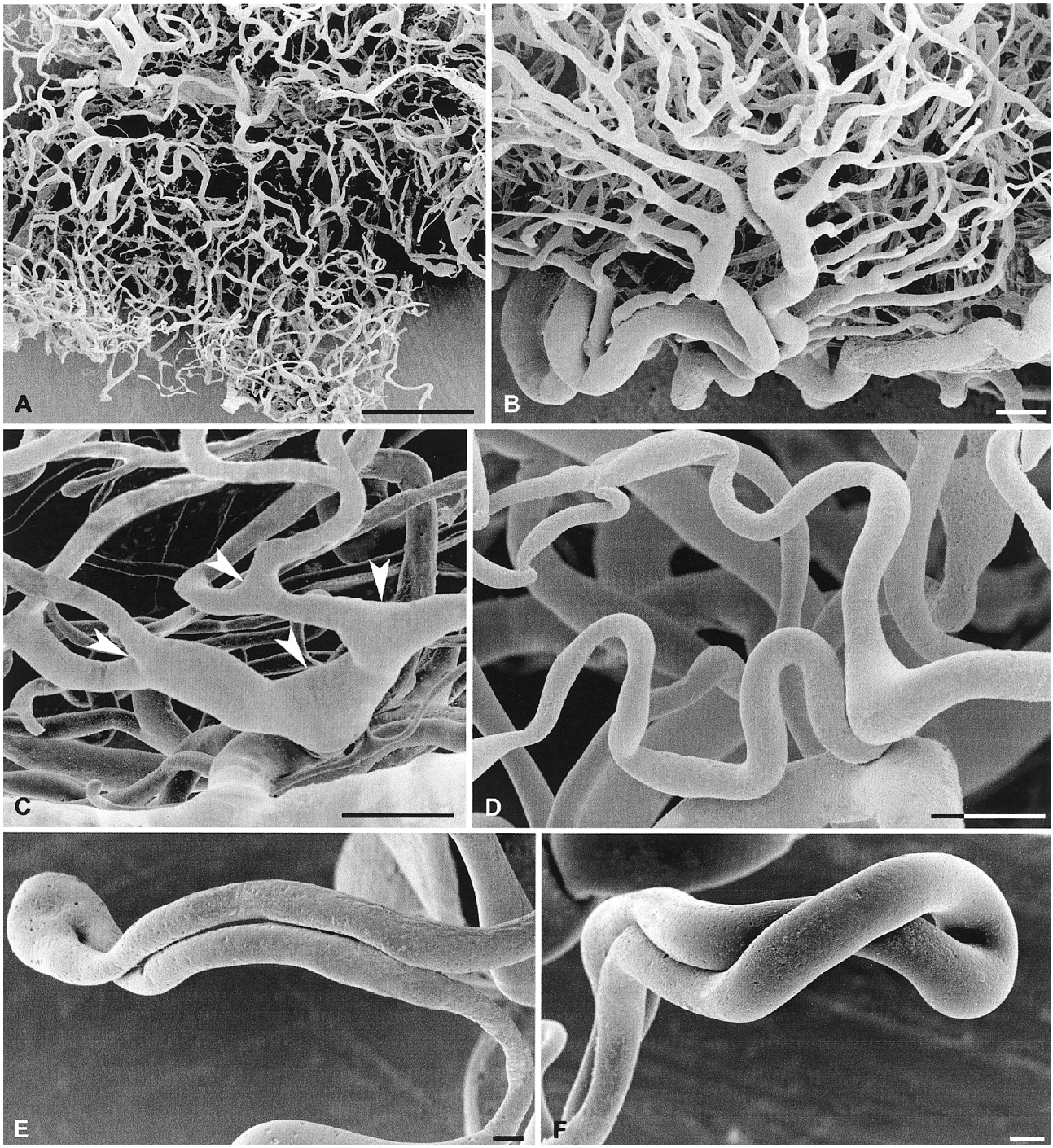

Fig. 3. – Scanning electron micrographs of microvascular corrosion casts of *Ambystoma* CNS. **A** : Dorsal view of the posterior telencephalon. The meningeal vessels are single elements in their winding course. Scale bar, 500 µm. **B** : The single meningeal vessels divide repeatedly in the lateral mesencephalic region. Scale bar, 100 µm. **C** : Parenchymal single vessels fork repeatedly (arrowheads) in the spinal cord. Scale bar, 100 µm. **D** : Wide U-shaped loops in single telencephalic vessels. Scale bar, 50 µm. **E** : A hairpin-like vascular structure of the medulla oblongata formed by closely apposed paired microvessels connected by a narrow U-shaped endloop. Scale bar, 10 µm. **F** : A mesencephalic vascular pair is bent and twisted. Scale bar, 10 µm.

DISCUSSION

Studies on the vertebrate CNS have revealed two main microvascular patterns. The most diffuse condition is represented by single vessels anastomozed in a complex three-dimensional network. It is found in eutherian mammals, turtles, fishes and anurans (Honma et al., 1987; Naka et al., 1987; Snyder et al., 1990a; Kleiter & Lametschwandtner, 1995). The second pattern is formed by paired microvessels dipping into the nervous tissue from the meningeal layer and terminally connected by a narrow U-shaped endloop. They constitute hairpin-like vascular structures that show no anastomotic connections with the adjacent vascular pairs. This condition is present in cyclostomes (Bundgaard, 1982) and widely represented in amphibians belonging both to the Gymnophiona (Craigie, 1940, 1941a) and the Urodela (Lazzari et al., 1991). As for reptiles, hairpin-like loops have been described in Sauria in studies with conventional light microscopy methods (Craigie, 1941b) and more recently by a TEM-SEM combination in Scincidae (Snyder et al., 1990b) and Lacertidae (Lazzari & Franceschini, 2000). The paired vessel pattern has also been pointed out in marsupials (Wislocki & Campbell, 1937; Bubis & Luse, 1964; Snyder et al., 1989).

A few other vertebrate species belonging to different classes have both single vessel meshwork and paired vessel loops in their CNS : the dipnoan *Epiceratodus forsteri* (Krefft, 1870) (Craigie, 1943), the crocodile *Alligator mississippiensis* (Daudin, 1802) (Craigie, 1941c) and *Ambystoma mexicanum* (Craigie, 1938, 1939; Bodenheimer & Brightman, 1968; Ciani et al., 1989). These studies are not recent, are mostly based on light microscopy techniques, and only the latest ones utilize TEM observations. As for *Ambystoma mexicanum*, these studies did not examine in detail the ultrastructural aspect of paired vessels compared to single ones, nor their distribution and the overall microvascular pattern of the CNS using SEM. The present study reports for the first time the ultrastructure and spatial organization of the vessels belonging to a model of CNS vascularization that is structurally intermediate between single vessel meshwork and paired vessel loops.

In *Ambystoma mexicanum* we found both single and paired vessels, but the first clearly prevailed. Their structural features identify them as capillaries. In particular, vascular pairs and single vessels show the same structure in transverse sections independently of their size, and no smooth muscle investment could be detected in TEM observations. This is in accordance with the condition of cyclostomes (Bundgaard, 1982) and urodeles (Bodenheimer & Brightman, 1968; Ciani & Franceschini, 1984), which have only paired vessels in their CNS. Marsupials and scincids, however, have hairpin-like capillary loops, which come off arterioles and venules that are themselves paired into the CNS (Snyder et al., 1989, 1990b). This different condition could be related to different CNS masses and, especially in marsupials, to the larger thickness and more complex neuronal organization of the brain.

In the CNS vascularization of *Ambystoma mexicanum*, age- and individual-based variations were not observed, particularly there was no difference between the vascular pattern in the CNS of a mature neotenic specimen and a metamorphosed one (Ciani, unpublished data).

Microscopic and submicroscopic observations showed that in *Ambystoma mexicanum,* hairpin-shaped loops do not branch into secondary ones. The simplest branching pattern, which is a secondary loop formed only by the contribution of one limb of a primary vascular pair, was found in Gymnophiona (Craigie, 1940), in *Triturus carnifex* (Lazzari et al., 1991), as well as in the lizard *Podarcis sicula* (Rafinesque, 1810) (Lazzari & Franceschini, 2000). This pattern is characterized by a very short secondary loop generally close to the terminal endloop. We hypothesize that this pattern relates to increased needs for blood supply in the tissue layer that is located in the deepest part of the neural wall next to the ependyma and brain ventricles. The other branching pattern, found in *Podarcis sicula* (Lazzari & Franceschini, 2000) and formed by the contribution of both capillary limbs of a primary pair, reminds us of marsupial hairpin capillary loops that nevertheless originate from closely paired intraparenchymal arterioles and venules (Snyder et al., 1989). Both branching patterns in the paired microvessel model meet major trophic demands of the neural tissue, and their absence in *Ambystoma mexicanum* could be related to trophic need satisfaction by the three-dimensional single vessel network.

According to Snyder et al. (1989) it is possible that paired capillaries play an important role in oxygen exchange in the nervous tissue by modifying diffusive gas conductance. These authors suppose that paired capillaries may operate as counter-current exchangers for carbon dioxide, the concentration of which in the blood becomes higher than normal levels. This could cause a large Bohr shift and release oxygen from the paired capillary blood. This event would raise the oxygen tension in the nervous tissue surrounding the vascular pair and increase the distance from the vascular pair to which the tissue could be adequately provided with oxygen. Nevertheless specific studies on gas exchange at the level of the paired capillaries of the CNS are still required to verify these assumptions.

In *Ambystoma mexicanum*, whose condition is intermediate between the capillary loop pattern and single vessel network organization, the closely related functional and phylogenetic significances are still unclear. According to Craigie (1938, 1940) the capillary loop condition represents an ancestral state with little adaptive significance, from which the single vessel network has developed. On the contrary, Wislocki & Campbell (1937) suggested a specific functional adaptation based on a counter current exchange process rather than a simple ancestral organization. Nevertheless Craigie (1938) considered it improbable that an adaptive evolution to paired brain microvessels would have occurred in various vertebrates differently located phylogenetically if it were not a return to a more primitive condition.

Snyder et al. (1989) compared the condition of a marsupial that has only paired vessels in its CNS, the northern native cat, *Dasyurus hallucatus* (Gould, 1842), with the condition of an eutherian mammal, the rat, which has only a single vessel network. These authors suggest that satisfying increased requests for gas exchange during

evolutionary development of the CNS may have required abandoning paired vessel pattern in favour of anastomotic meshwork. In this regard, *Ambystoma*, showing both vascular patterns and particularly with the anastomotic meshwork prevailing on the paired vessel loops, would present a CNS vascularization more evolved than the condition of Gymnophiona and most Urodela, which show only paired vessel loops. Moreover the condition of *Ambystoma* would be less evolved than the anuran one, which shows only single vessels in the CNS.

The present study shows that the structure and organization of hairpin-shaped paired vessels of *Ambystoma mexicanum* are the same as in vertebrates that have the pair pattern exclusively : cyclostomes (Bundgaard, 1982), gymnophiones (Craigie, 1940, 1941a), urodeles (Bodenheimer & Brightman, 1968 ; Ciani & Franceschini, 1984), lizards (Snyder et al., 1990b ; Lazzari & Franceschini, 2000) and marsupials (Bubis & Luse, 1964 ; Snyder et al., 1989). Moreover in newts (Ciani & Franceschini, 1984 ; Lazzari et al., 1991), lizards (Lazzari & Franceschini, 2000), and marsupial northern native cat (Snyder et al., 1989), as well as *Ambystoma mexicanum* (present study), the two paired limbs appear closer than in opossum CNS in which cells of unknown origin and function separate the two elements of a loop (Bubis & Luse, 1964). The most important feature of *Ambystoma mexicanum* paired vessels and of the vertebrates that possess only paired vessels in their CNS (cyclostomes, gymnophiones, newts, lizards and marsupials) is that the perivascular glial processes never enter into the thin intercapillary wall that separates the two adjacent limbs (Bubis & Luse, 1964 ; Bodenheimer & Brightman, 1968 ; Bundgaard, 1982 ; Ciani & Franceschini, 1984). The fact that both vascular loops and single vessels are found in all regions of the CNS would indicate that the functional needs that promoted the transition from paired vessel pattern to anastomotic meshwork operated in the whole CNS.

Many studies on various vertebrate species showed that brain capillaries are involved in the blood-brain barrier (see Brightman, 1992 ; Brightman & Tao-Cheng, 1993 ; Vorbrodt, 1993). Its main structural bases are the lack of fenestration, general absence of free transendothelial passages, and, particularly, the presence of tight junctions sealing adjacent endothelial cells and preventing the diffusion of substances such as electron microscopic tracers. Pits of the endothelial plasmalemma and vesicular profiles in the cytoplasm may be involved in transcytosis through the endothelial cells (Pardridge, 1993). Even though direct functional studies on vascular permeability to microscopical tracers and histochemical localization of enzyme activities have not yet been carried out in the brain capillaries of *Ambystoma mexicanum*, the ultrastructural features here reported are in accordance not only with the condition of *Triturus carnifex* (Ciani & Franceschini, 1984) and lizards (Shivers, 1979 ; Lazzari & Franceschini, 2000), which show a paired vessel pattern, but also with the findings in eutherian mammals (Brightman, 1992), which have a three-dimensional brain capillary network.

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Massinium, a new genus in the family Phyllophoridae (Echinodermata : Holothuroidea : Dendrochirotida) with description of a new south-west Indian Ocean species *M. maculosum*

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ABSTRACT. Several specimens of a phyllophorid holothuroid collected from off the KwaZulu-Natal coast, Republic of South Africa, represent a species new to science. In the structure of the calcareous ring and the absence of tables from the body wall it resembles *Neothyonidium magnum* and *N. arthroprocessum*. In all three species the posterior processes of the calcareous ring are distally joined. Hence, they do not correspond to the diagnosis of *Neothyonidium* as defined by its type species, *N. hawaiense*, and are now assembled in the new genus *Massinium.* The new species is described as *Massinium maculosum.* The three species are keyed and a distribution map is provided.

KEY WORDS : Holothuroidea, Phyllophoridae, *Massinium* gen. nov., *Massinium maculosum* sp. nov., taxonomy

INTRODUCTION

The genus *Neothyonidium* was erected by Deichmann (1938) for phyllophorids with 20 tentacles positioned in two well-defined circles and a calcareous ring with long posterior prolongations, formerly classified in *Thyonidium* Düben & Koren, 1844 and *Phyllophorus* Grube, 1840. Unfortunately, Deichmann (1938) refrained from giving a complete diagnosis of the genus. Instead, the character of the genus was covered by only two phrases in a key to the Phyllophoridae of the eastern Pacific ['Tentacles arranged in an external circle of five pairs of large tentacles and an inner circle of much smaller tentacles, five deeply divided or five pairs' and 'Calcareous ring tall, tubular with long deeply divided posterior prolongations and long narrow interradials' (Deichmann 1938 : 379)]. Only a single species was included by Deichmann (1938) : *N. hawaiiense* (Fisher, 1907), the designated type species by monotypy.

Heding & Panning (1954) followed Deichmann (1938) in recognizing the systematic value of the calcareous ring in the classification of phyllophorids. These authors elaborated Deichmann's (1938) diagnosis of the genus *Neothyonidium* by adding two-pillared tables as an additional diagnostic character. By doing so, Heding & Panning (1954) recognised *Neothyonidium hawaiiense* (Fisher, 1907) as type species of the genus, to which they also assigned *Phyllophorus inflatus* Sluiter, 1901; *P. dearmatus* Dendy & Hindle, 1907; *P. intermedius* Koehler & Vaney, 1908; *P. vultur* Sluiter, 1914; *P. minutus* Ohshima, 1915; *Thyonidium magnum* (Ludwig, 1882) and, with doubt, *P. parvus* (Ludwig, 1881). However, they downgraded *T. alexandri* Fisher, 1907 as a synonym of *N. hawaiiense*. Unfortunately, the descriptions given by Heding & Panning (1954) are in too many cases largely dependent on the accounts of earlier writers. Since then, Pawson (1965) described *N. armatum* from New Zealand; Cherbonnier (1988) *N. dissimilis* from Madagascar; Rowe (1989) *N. parvipedum* from north-eastern Tasmania; Thandar (1989) *N. arthroprocessum* from South Africa and Liao & Pawson (2001) *N. spiniferum* from China. In addition, Rowe (in Rowe & Gates 1995) resurrected *T. alexandri* from the synonymy of *N. hawaiiense* as a valid species. A critical review of the genus *Neothyonidium* is beyond the scope of this paper, but it is safe to state that several species assigned to it may not belong to this genus and others may be conspecifics of some well-known species.

Thandar (1989) emended the diagnosis of the genus *Neothyonidium* to accommodate the southern-African *N. arthroprocessum*, characterised by its peculiar U-shaped deposits in the body wall. At that time he justified this inclusion by stating that the rods of *N. arthroprocessum* may be table derivatives. Later on, additional records of *N. arthroprocessum* revealed that these deposits are derived from minute buttons rather than from tables (Thandar 1996). On the basis of a new species of a phyllophorid holothuroid – here described from the east coast of South Africa – characterised by possessing only rosette-shaped granules and "pseudobuttons" in the body wall, it became clear that the emended diagnosis of *Neothyonidium* (Thandar 1989; see also 1996) could not be upheld. In fact, both *N. arthroprocessum* and the new species belong to another natural group without body wall tables. Moreover, a careful study of the calcareous ring, internal morphology and ossicle assemblage revealed that

these two species, together with the tropical west Pacific *N. magnum* (Ludwig, 1882), constitute a well-defined lineage, not corresponding with the diagnosis of *Neothyonidium* as defined by its type species, excellently described by FISHER (1907). This lineage thus constitutes a new genus, here diagnosed as *Massinium*, characterised by the following synapomorphies : a complex calcareous ring with the posterior processes distally linked to each other; four polian vesicles and a body wall that is always devoid of tables. Moreover, in *N. arthroprocessum* and in the new species, the respiratory trees are extremely complex, with a richly-branched subsidiary trunk to each tree. Unfortunately, since we have only anterior fragments of *N. magnum* at our disposal, the detailed structure of its respiratory trees remains poorly described. The new species is here described as *M. maculosum*.

MATERIAL AND METHODS

During recent surveys of the echinoderm fauna of KwaZulu-Natal (Republic of South Africa), several specimens belonging to a species new to science were found. Collecting was done by SCUBA-diving to depths of a maximum of 44 m, but specimens belonging to the new species were found between 17 and 20 m. Specimens were anaesthetized in 5% magnesium chloride for 4 hours, transferred to 100% buffered alcohol for 24 hours, and then to 70% buffered alcohol for permanent storage. They were studied according to conventional methods outlined by workers such as FISHER (1907), DEICHMANN (1948), ROWE & DOTY (1977) and MASSIN (1999), amongst others. Ossicles were removed in household bleach, washed in two changes of distilled water and illustrated with the camera lucida. For scanning electron microscopy, the ossicles were passed through two changes of absolute alcohol and transferred with a fine pipette together with a little alcohol onto a specimen stub to which they normally stick once the alcohol evaporates. They were then sputter-coated with gold for 5 minutes at 30-40 mA and photographed with a Philips SEM 500.

Museum acronyms

MRAC	Muséum Royal de l'Afrique centrale, Tervuren, Belgium
NHM	Natural History Museum, London, United Kingdom
SAM	South African Museum, Cape Town, Republic of South Africa
IRSNB	Institut royal des Sciences naturelles de Belgique, Brussels, Belgium

RESULTS

Family PHYLLOPHORIDAE Oestergren, 1907
Subfamily Semperiellinae Heding & Panning, 1954

Genus *Massinium* gen. nov.

Diagnosis. Dendrochirotid holothuroids with twenty tentacles arranged in two circles of 10 + 10; anus encircled by calcareous teeth; calcareous ring complex, elongated, tubular, with both radial and interradial plates fragmented into a mosaic of small pieces, prolonged posteriorly with posterior processes distally-linked to adjoining processes of neighbouring plates forming a ribbon-like structure beneath the water-vascular ring; Polian vesicles large, four; ossicles of body wall comprise rods or rosette-shaped deposits, tables always absent; introvert with two-pillared tables and numerous rosettes and/or rods.

Type species *Massinium maculosum* sp. nov.

Etymology. This genus is named after Dr. Claude Massin of the Royal Belgian Institute of Natural Sciences, Section Malacology, in recognition of his excellent contributions to the taxonomy of holothuroids. The gender is neuter.

Key to the genus *Massinium*	
1	Body wall ossicles comprise solely rods of variable form, never rosette-like, with minute, often occluded perforations at ends; introvert with two-pillared tables, rods and rosette-shaped bodies . . . . . ***M. arthroprocessum*** **(Thandar, 1989)**
1'	Body wall ossicles comprise only rosette-like bodies **2**
2	Outer tentacles devoid of ossicles; introvert with tables and rosettes; body colour off-white to grey, mottled liberally with dark brown; tentacles with white shaft dashed with brown, tentacle tips spotted with yellow and white . . . . . ***M. maculosum*** **sp. nov.**
2'	Ossicles of outer ring of tentacles include rosettes; introvert with tables only; body colour yellow to brown, tentacles darker brown . . . . . ***M. magnum*** **(Ludwig, 1882)**

Massinium maculosum sp. nov.
(Figs 1- 5)

Diagnosis. Medium-sized, U-shaped phyllophorid with bloated mid-body and relatively short anterior and posterior ends. Anus surrounded by teeth and papillae, variously developed. Ossicles of body wall short, thick, flat, oval to round rosette-shaped rods and "pseudobuttons", often with minute perforations. Introvert ossicles include tables and rosettes. Tentacles of outer ring without ossicles, tentacles of inner ring with rods and rosettes.

Etymology. The name *maculosum* refers to the conspicuous colouration of the whitish shaft of the tentacles, mottled with dark brown and yellow.

Name bearing type. Holotype SAM A27882 (specimen with detached calcareous ring)

Type locality. Mabibi, KwaZulu-Natal Republic of South Africa (Fig. 5).

Material examined. Holotype (specimen with detached calcareous ring), RSAKZN/01122, SAM A27882, 13.ii.2001, 20 m, SCUBA-diving, Y. Samyn. Paratype 1 (calcareous ring only), RSAKZN/01121(a), SAM A27883, other data same as holotype. Paratype 2 (calcareous ring only), RSAKZN/99217, SAM A27884, 17.viii.1999, Sodwana Bay (2-Mile Reef), 14 m, SCUBA-diving, Y. Samyn & P. Timm. Paratype 3 (specimen with calcareous ring attached), RSAKZN/01121(b), MRAC 1701, other data same as holotype. Paratype 4 (calcareous ring only), RSAKZN/99139, MRAC 1702, 12.viii.1999, Sodwana Bay (9-Mile Reef), 17 m, SCUBA-diving, Y. Samyn. Paratype 5 (specimen devoid of calcareous ring), RSAKZN/01124, NHM 2003.570, other data same as holotype. Paratype 6 (calcareous ring

Fig. 1. – *Massinium maculosum* sp. nov. as photographed after removal from the substrate at Sodwana Bay, 2-Mile Reef. (Picture by R. Anderson).

only), RSAKZN/01123, NHM 2003.571, other data same as holotype.

Description. Holotype (Fig.2A). Specimen entire, well-preserved but with ventral surface slightly ruptured and calcareous ring detached. Body form cylindrical, somewhat U-shaped, with the mid-body bloated and with narrower anterior and posterior ends. Length along ventral surface 150 mm, height of mid-body 41 mm, anterior and posterior ends 30 mm and 40 mm in length and 11 mm and 17 mm in diameter respectively. Colour light yellow to off-white, mottled with dark brown, more pronounced at the ends. Body wall thin and smooth in bloated part; thick and wrinkled proximally and distally. Body wall podia papilliform, scattered, more numerous proximally and distally, with dark narrow rings encircling reduced suckers. Tentacles 20, in two circles of 10+10, well branched, outer tentacles with whitish stalks spotted with dark brown, branches black proximally and white at tips, inner tentacles much shorter, also with whitish stalks and uniformly black branches. Largest tentacle about 30 mm long. Anus encircled by five groups of papillae, with 4-7 papillae per group. Single papilla of one group heavily calcified, appearing as a tooth covering most of the anal opening; another tooth underdeveloped, not covering anus.

Calcareous ring (see that of paratype 4 : Fig. 4H) 42 mm in length, distinctly tubular with radial and interradial plates fused for three-quarters of their length, both radial and interradial plates broken into a mosaic; radial plates anteriorly bifid, with depressions for attachment of retractor muscles; anterior of interradial plates pointed. Posterior processes of radial plates short, broad, bifurcating and uniting with adjacent processes of neighbouring plates to form a ribbon-like structure encircled by the water vascular ring. Polian vesicles four (one per interradius except the mid-dorsal), up to 25 mm long. Stone canal single, slightly shorter than polian vesicles, free, terminating in an ovoid, well-calcified madreporic body. Gonad (testis) in two tufts, well-developed, white, branched, mostly dichotomously. Respiratory trees remarkably well-developed, reaching anterior end of body, with heavily branched subsidiary trunks extending both to the level of the calcareous ring and beneath the cloaca; exact origin and ramifications of the subsidiary branches could not be determined without further damage to the holotype.

Ossicles of the dorsal and ventral body wall similar – comprising small, thick, flat, elongated to roundish bodies in the form of rods, rosettes and "pseudobuttons", the latter evidently derived from rosettes (Fig. 2B, C, 3A-C). Elongate ventral deposits 27-40 µm long (mean 34.5 µm) and 13-24 µm wide (mean 19.5 µm). Roundish ventral deposits 24-42 µm long (mean 28.6 µm) and 18-41 µm wide (mean 25.8 µm). Elongate dorsal deposits 27-45 µm long (mean 35 µm) and 14-27 µm broad (mean 17.8 µm). Roundish dorsal deposits fewer, 23-25 µm long (mean 24.7 µm) and 19-23 µm wide (mean 22 µm). Rosettes of dorsal body wall more abundant and more complex. Some ossicles large and bone-shaped, dorsally 26-51 µm long (mean 39 µm) and 6-14 µm wide (mean 8.3 µm); ventrally 37-49 µm long (mean 45 µm) and 6-11 µm wide (mean 8.6 µm). "Pseudobuttons" oval, irregular, multilocular, more dominant ventrally, holes minute, larger in ventral body wall (Fig. 3D, E). Ventral podial deposits in the form of tables, plates, rods and rosettes similar to those of body wall (Fig. 2F). Tables with smooth ovoid discs, 50-75 µm long, perforated by four large central holes and a variable number of irregularly-positioned peripheral ones. Spires of moderate height, comprising two pillars united by a single cross bar or cross bar absent, pillars diverging distally to terminate in two spiny tips. Rods straight, smooth, elongate, with terminal perforations and/or processes. Some rods irregular with medial processes or formed into plate-like deposits with several perforations in the middle and/or the tips. End-plates present with numerous holes, not varying in size peripherally (Fig. 4G). Dorsal podia without tables; other deposits similar to those of ventral podia. Introvert podia with tables and rods (Fig. 4B). Anal podia with tables and rosettes (Fig. 4E, F). Large tentacles (from outer ring) without deposits. Small tentacles (from inner ring) comprise tables at their roots, similar to those of introvert, rosettes and slender rods at their tips; rods straight or slightly curved with wrinkled apices (Fig. 4C, D). Introvert deposits comprise tables and rosettes (Figs 2D, E; 3F, G). Tables with large subcircular to oval discs with smooth margins and perforated by four large central holes and usually a single circle of smaller holes, the latter 3-12 in number. Table discs 69-135 µm long (mean 100 µm) and 55-104 µm wide (mean 79 µm); spire 30-53 µm high (mean 45 µm), with a single cross bar and 2-6 terminal teeth. Occasionally pillars reduced to knobs on surface of disc. Rosettes similar to those of body wall, 23-53 µm long (mean 37 µm).

Paratype 1. Represented by calcareous ring and tentacles only. Introvert bordered by numerous podia arranged in rows in the radii. Tentacles as in holotype, longest 24 mm. Polian vesicles four, of unequal length, longest 29 mm. Stone canal free, slightly twisted proximally. Madreporite poorly calcified, slightly wider than stone canal.

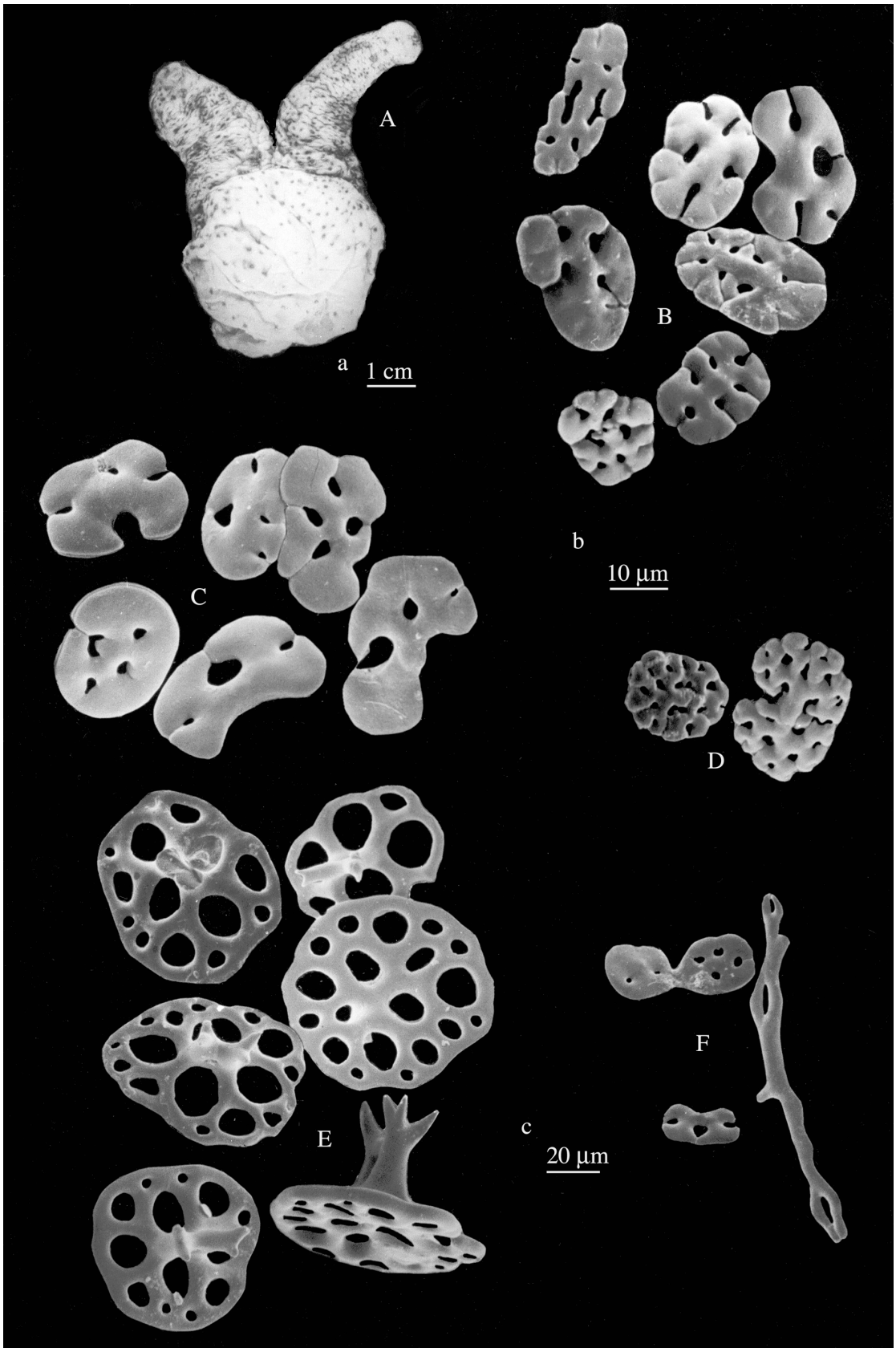

Fig. 2. – *Massinium maculosum* sp. nov. A, entire; B, rosette-shaped ossicles from mid-dorsal body wall; C, rosette-shaped ossicles from mid-ventral body wall; D, rosettes from the introvert; E, tables from the introvert; F, podial deposits.

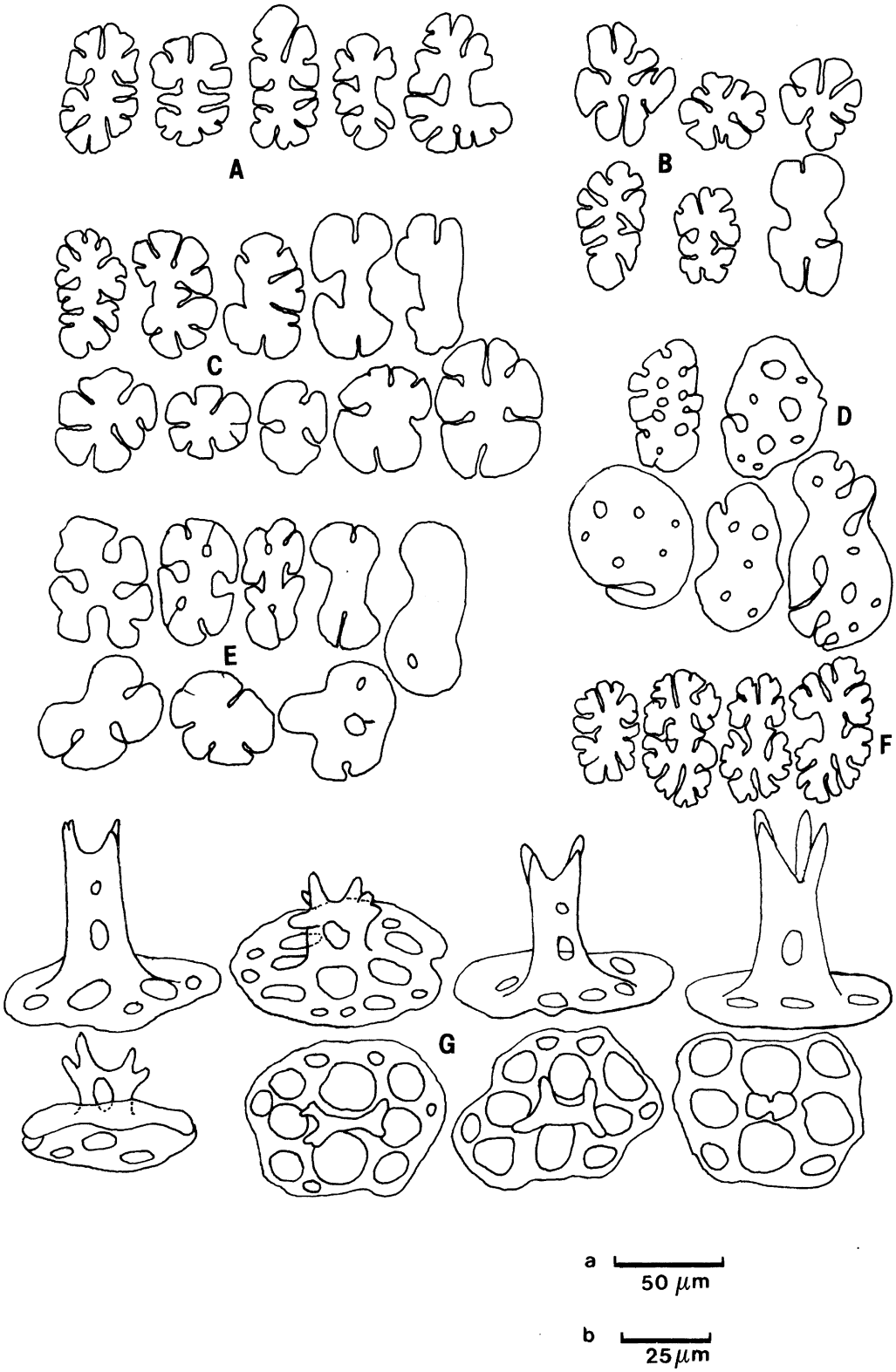

Fig. 3. – *Massinium maculosum* sp. nov. Ossicles. A, rosette-shaped deposits from antero-dorsal body wall; B, rosette shaped plates from antero-ventral body wall; C, rosette-shaped deposits from mid-dorsal body wall; D, pseudobuttons from dorsal body wall; E, pseudobuttons from ventral body wall; F, Rosettes from introvert; G. tables from introvert. (A-F - scale b; G - scale a)

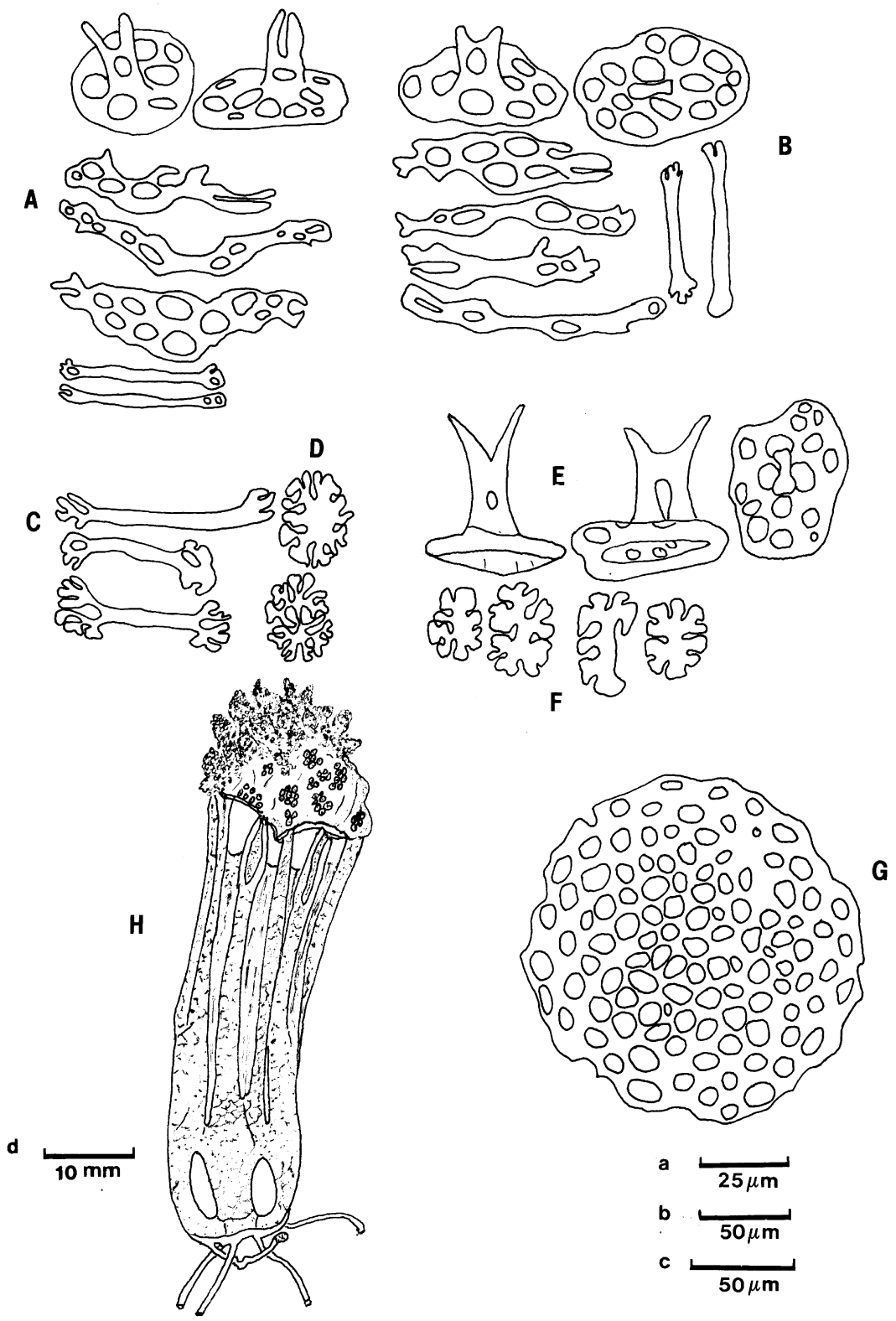

Fig. 4. – *Massinium maculosum* sp. nov. Calcareous ring and ossicles. A, introvert deposits; B, introvert podial deposits; C, rods of tentacle of inner ring; D, rosettes of tentacle of inner ring; E, tables from anal podia; F, rosettes from anal region; G, end-plate from pedicel; H, calcareous ring of paratype 4 showing tentacles, four polian vesicles and a single stone canal.
(A, B & E – scale c; C, D & F – scale a; G – scale b & H – scale d)

Paratype 2. Represented by calcareous ring, tentacles and anterior fragment of body wall. Polian vesicles four, two branched proximally and one terminally, giving the impression that there are more than four polian vesicles. Stone canal typical but very much coiled proximally. Madreporite as in holotype. Body wall ossicles typical. Ossicles absent from large tentacles. Small tentacles include rods, rosettes and tables, table discs subcircular to oval with four large central perforations and numerous smaller perforations, up to 20, in one or two circles. Spire high, consisting of two pillars and one or two cross-bars, terminating in four teeth. Rosettes similar to those of body wall. Rods straight or curved, with one to two terminal perforations.

Paratype 3. Same form and colouration as holotype, with eviscerated calcareous ring still attached to main body. Length along ventral surface 130 mm, height of mid-body 32 mm, anterior and posterior ends 20 mm and 9 mm respectively. Anus encircled by four calcareous teeth of which one is best developed, each bordered by three to four papillae. Calcareous ring as in holotype, fragmented. Polian vesicles four, elongate, up to 37 mm, tubular. Stone canal free, slightly twisted proximally, madreporite hardly distinguishable from stone canal. Tentacles as in holotype, number difficult to determine as inner circle is obscured within oral cavity. Length of larger tentacles variable, up to 20 mm. Ossicles of ventral and dorsal body wall similar to holotype, 33-59 µm long (mean 46 µm); introvert tables with discs 104-137 µm long (mean 114 µm).

Paratype 4 (Fig. 4H). Represented by the calcareous ring and tentacles as illustrated. Calcareous ring 50 mm in length, best preserved of all type material.

Paratype 5. Specimen small without calcareous ring. Colour as in holotype but dark speckling more pronounced, obscuring the lighter areas of the skin. Length along ventral surface 75 mm, height of mid-body 21 mm, anterior and posterior ends 15 mm and 9 mm respectively. Five equally-sized anal teeth, each bordered by four papillae. Body wall ossicles similar to holotype, 31-60 µm long (mean 40 µm).

Paratype 6. Represented only by calcareous ring and a fragmented part of the body wall. Four polian vesicles, single free stone canal, proximally coiled. Madreporite ovoid, slightly wider than stone canal.

Ecology. This species lives buried deeply in sand, under boulders and coral debris exposing its tentacle crown only when feeding. Upon slightest disturbance, even shading, the tentacle crown and the introvert are swiftly retracted into the body leaving no trace of the animal. Individuals were observed to be actively feeding during daytime (recorded observations : 11h00-13h45; no observations were made at night). The species appears to be more common at Mabibi where, during 108 dive-minutes, six individuals were spotted whereas at Sodwana, only two individuals were spotted during the same period of time at the 9-Mile Reef and just one at the 2-Mile Reef, in more than nine hours of underwater observations. Depth range 14-21 meters.

Geographical distribution. For now only known from northern KwaZulu-Natal (Republic of South Africa) (Fig.5).

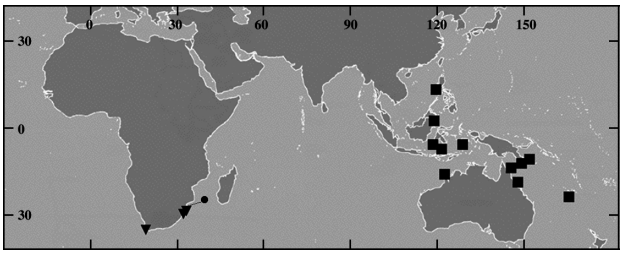

Fig. 5. – Distribution of the currently known *Massinium* spp. : *Massinium arthroprocessum* (Thandar, 1989) is represented by triangles; *M. maculosum* sp. nov. by a circle; *M. magnum* (Ludwig, 1882) by squares.

DISCUSSION

Massinium maculosum is very similar to *Massinium magnum* (Ludwig, 1882), differing in colouration of the tentacles, the absence of ossicles from the tentacles of the outer ring and the form of the body wall deposits. The original description of *M. magnum* was based only on the introvert and the calcareous ring. Only Sluiter (1901), Domantay (1933) and Cherbonnier (1980) claim to have studied complete specimens of this species. However, Cherbonnier (1980), who also examined Ludwig's (1882) type, is of the opinion that both Sluiter's and Domantay's specimens may not be the true *M. magnum* as there are significant differences in the form of the introvert tables – those of the holotype are according to him 'absolument identiques' [sic] to his specimens in having multilocular discs with one or more series of peripheral holes in contrast to Sluiter's and Domantay's specimens, which have fewer peripheral perforations, restricted in an incomplete or a single series. Sluiter (1901) unfortunately failed to describe the colour of his specimens; Domantay (1933), on the other hand, described the colouration of his specimens accurately. The latter author noted that the tentacles are deep chocolate brown in life and almost black in contracted condition; the introvert is brown and the body wall is almost white. We have examined the introvert in specimens of *M. magnum* described by Massin (1999) from Sulawesi (Indonesia) and also in two undescribed specimens collected by Massin from Papua New Guinea. As stated by Massin (1999) the introvert of his Sulawesi specimens contains only tables. We here corroborate Massin's (1999) observations and further state that this is also true for his Papua specimens. However, the introvert tables of the Sulawesi specimen have tall spires and few peripheral holes, many of which alternate with the large central holes; whereas those of the Papua specimens have moderate to low spires and more peripheral holes. It is therefore certain that *M. magnum* is either a highly variable species or a species-complex. This is corroborated by colour illustrations of *M. magnum* given by Féral & Cherbonnier (1986) from New Caledonia, Gosliner *et al.* (1996) from Papua New Guinea and Massin (1999) from Sulawesi. Hence a redescription of *M. magnum*,

based on all the available material, is urgent and will the subject of our next investigation.

In his revision of the southern African phyllophorids, THANDAR (1990) listed only *M. arthroprocessum* in the subfamily Semperiellinae. The new species now increases to two the number of species in this subfamily occurring in southern Africa.

Massinium is distributed throughout the tropical and subtropical Indo-West Pacific Ocean : *M. magnum* is known from the tropical West Pacific, whereas *M. arthroprocessum* and *M. maculosum* for now are restricted to the subtropical West Indian Ocean. The distribution is mapped in fig. 5.

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Dwarf spiders (Erigoninae, Linyphiidae, Araneae) : good candidates for evolutionary research

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ABSTRACT. Males of numerous erigonine dwarf spider species, including those in the genera *Oedothorax* and *Diplocephalus*, are characterised by elaborate structures on the head region. Three evolutionary hypotheses for these head structures are : lock-and-key (reproductive isolation) hypothesis, "conflict of interest hypothesis" and diverse sexual selection hypotheses.

Oedothorax gibbosus (Blackwall, 1841) is a dwarf spider characterised by male dimorphism; the *gibbosus* morph has a hunch on the last third of the carapace, anterior to which is a hairy groove; the *tuberosus* morph does not have these features. During the so-called gustatorial courtship the female inserts her chelicerae into the hairy groove of *gibbosus*.

Species recognition experiments reveal the occurrence of interspecific homo- and heterosexual "gustatorial courtships" of the female as well as the male of the closely related species *Oedothorax fuscus* (Blackwall, 1834) towards the *gibbosus* male. These interspecific courtships can be interpreted as robbery of the nuptial gift located in the groove and the hunch of the *gibbosus* males. *Gibbosus* males can also rob the nuptial gift of each other, but this occurs only rarely. We have never observed a 'gustatorial robbery' between a *tuberosus* male and a *gibbosus* male. There are also interspecific interactions between a *tuberosus* male and an *O. fuscus* female suggesting poorly developed reproductive isolation between these sister species.

These interspecific courtships are in disagreement with the lock-and-key hypothesis. Indeed, according to this hypothesis the head structures of erigonine males should function as an early prevention of hybridisation. Female chelicerae and male head structures thus do not operate as key and lock. Therefore, the head structures might have evolved under the influence of sexual selection.

KEY WORDS : Araneae, Erigoninae, speciation hypotheses, head structures, interspecific courtships, nuptial feeding.

INTRODUCTION

Many publications have addressed the evolution of genitalia. Spiders (Eberhard, 1997; Huber, 1996, 1999), water striders (Arnqvist et al., 1997; Arnqvist & Thornhill, 1998) and other insects (Arnqvist, 1997, 1998; Eberhard, 1997; Elgar, 1998; Arnqvist et al., 2000) have recently become preferred model organisms for such research. Spectacular morphological diversification of genitalia is widespread among animals with internal fertilisation. Even in closely related taxa, genitalic morphology typically differs greatly between species (Eberhard, 1985). There are three main general hypotheses for the evolution of animal genitalia (Eberhard, 1985, 1990, 1993, 1996; Arnqvist, 1997) : the lock-and-key hypothesis (selection for pre-insemination reproductive isolation : specific and unique male intromittent genitalia -the key- fit in female genitalia -the lock), the sexual selection hypothesis (relationship between genitalic morphology and relative post-copulatory fertilisation success) and the pleiotropy hypothesis (genitalic evolution is an indirect result of evolution of genetically-correlated characters, via pleiotropic effects of genes that code for both genitalic traits and evolving general morphology (Mayr, 1963; Arnqvist et al., 1997)).

Arnqvist (1997) reviews the different suggestions of how genitalia might evolve through sexual selection : (1) cryptic female choice (Eberhard, 1985), (2) sexual conflicts (Thornhill, 1984) and (3) sperm competition (Smith, 1984).

The evolution of non-genitalic contact structures is also very interesting. Huber (1999) reviews the evolutionary hypotheses useful for such non-genitalic contact structures; he applied the hypotheses to the artful chelicerae of male pholcid spiders (Araneae, Pholcidae). In that publication the author considered again the lock-and-key hypothesis (reproductive isolation hypothesis) and different sexual selection hypotheses (male-male competition (Eberhard & Briceno, 1985) and sexual selection by female choice (Eberhard, 1985)), but also the "conflict of interest hypothesis" ("genitalic arms race" between the sexes relating to physical coercive mating) (Alexander et al., 1997) and the "sperm holder hypothesis" (pholcid chelicerae may function to hold the sperm during sperm uptake) (Brignoli, 1973). By elimination, Huber (1999) determined that cryptic female choice is the hypothesis that best fits his data. The theory of sexual conflict is the subject of several other empirical (Arnqvist, 1998; Arnqvist et al., 2000; Arnqvist & Rowe, 2002) and theoretical studies (Gavrilets, 2000).

Several publications confirm that sexual selection is more and more regarded as having the potential to play a major role in speciation (Arnqvist & Nilsson, 2000; Panhuis et al. 2001). According to Arnqvist (1998), genitalic evolution is more than twice as divergent in groups in which females mate several times than in groups in which females mate only once. Masta & Maddison (2002) provide genetic, behavioural and simulation data that illustrate that the striking and possibly recent divergence in traits of male behaviour and morphology among populations of the jumping spider *Habronattus pugillis* Griswold, 1987 can be attributed to sexual selection.

Males of erigonine spiders and other spider species are characterised by the occurrence of elaborate structures on the head. These have been analysed morphologically in several species (Lopez, 1976, 1987; Meijer, 1976; Blest & Taylor, 1977; Vollrath, 1977; Lopez & Emerit, 1981; Huber, 1997; Heinemann & Uhl, 2000; Hormiga, 2000; Schaible et al., 1986; Schaible & Gack, 1987; Schlegelmilch, unpubl. data). Schaible et al. (1986) suggested that the primary function of the male head structures in these erigonine spiders is to fix the position of the female during copulation. These authors assume that the exocrine glands associated with these head structures produce secretions, which females ingest during courtship and/or copulation. Schaible et al. (1986) were the first to suggest that the cephalic structures probably secrete a fluid that is important for the so-called gustatorial courtship, being the uptake of secretions by the female from a body part of the male during courtship. These cephalic structures can be seen as non-genitalic contact structures. Huber (1997) mentions a similar case of a non-genitalic contact structure, namely the frontal lobe in male *Modisimus culicinus* (Simon, 1893).

Oedothorax gibbosus (Blackwall, 1841), an erigonine spider, is special because males are dimorphic. One morph, the *gibbosus* male, has cephalic structures, namely a hunch on the last third of its carapace anterior to which is a hairy groove (Fig. 1); the *tuberosus* morph on the other hand does not have these cephalic features. The large number of different gland cells in the hunch of *gibbosus,* in comparison with the few gland cells in the cephalic region of *tuberosus* (Vanacker, unpubl. data), is certainly an indication that *gibbosus* males not only secrete pheromones, but also a nuptial gift. During the gustatorial courtship, which evidently can only be performed by *gibbosus* males, the female puts its chelicerae into the hairy groove of *gibbosus* and exhibits a feeding behaviour. Because of this nuptial gift it can be assumed that *gibbosus* is sexually more attractive to females (Vanacker et al., in press).

After the courtship copulation follows. Starting from a face-to-face position, the male shifts its cephalothorax underneath that of the female. In this way the two palps can easily reach the epigyne. During a palp insertion, sperm is pumped into the epigyne by means of swelling and shrinking of the haematodoch. This last structure is a bladder and is a part of the male palp. The transition of gustatorial courtship to copulation can happen smoothly or there can be a break between courtship and copulation. During copulation most females remove their chelicerae from the hairy groove of *gibbosus.* Fixation of the position (Schaible et al., 1986) is thus not the most important function of the male head structures of the *gibbosus* morph male.

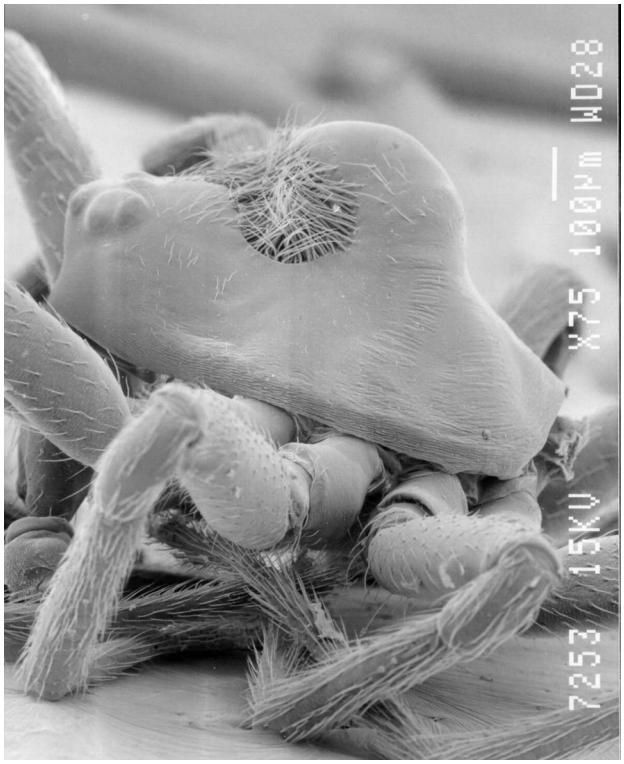

Fig. 1. – Scanning electron micrograph of a *gibbosus* male, showing the hunch and the hairy groove on the carapax

What is now the function of the different cephalic structures of the erigonine spiders? Are they used as a pre-copulatory species-recognition mechanism or as a signal in a context of sexual selection (sexual conflict) important for e.g. partner choice by the female? In order to answer these questions we did some species-recognition experiments with *Oedothorax gibbosus* and *Oedothorax fuscus* (Blackwall, 1834) and made some unexpected observations. On the basis of these experiments we also try to determine whether *gibbosus* males are sexually more attractive than *tuberosus* males.

MATERIAL AND METHODS

Oedothorax gibbosus spiders were caught in an oligo- to mesotrophic alder marsh, the typical habitat of this dwarf spider species, in the public nature reserve "Het Walenbos" at Tielt-Winge, 30 km north-east of Brussels, Belgium; *O. fuscus* spiders were collected in the military domain at Nieuwpoort, Belgium. The *O. gibbosus* dwarf spiders were caught by hand on August 4 2001, October 13 2001, October 27 2001 and July 5 2002; the *O. fuscus* spiders on October 31 2001 and January 22 2003. The spiders used in the experiments were descendants of female spiders inseminated in the field or were obtained by laboratory crossing of a female with a *tuberosus* or a *gibbosus* male. All spiders were kept separately in small plastic vials (5 cm diameter and 2.5 cm height) with a thin bottom of plaster, in a climatic chamber at a photoperiod L:D 16:8 and a temperature of 20˚C. Before the second moult they were fed with four *Sinella curviseta* springtails each day; after the second moult they received three fruit flies per day. The vials were moistened regularly to maintain a relative humidity near 100%.

In a first series of experiments we examined the different interactions between a *gibbosus* male, an *O. gibbosus* female and a male or female *O. fuscus* spider. In the fifth of that series of experiments we used seven *O. fuscus* males. In the sixth experiment we used another *gibbosus* male instead of *O. fuscus* spiders, to study whether there would be an interaction between the two *gibbosus* males. We also did analogous experiments with a *tuberosus* male, an *O. gibbosus* female and one *O. fuscus* spider. The *gibbosus*/*tuberosus* male and the *O. fuscus* spider(s) were put in the vial of the *O. gibbosus* female. This female had inhabited the vial since the first juvenile instar and had already produced a web, which is necessary for the gustatorial courtship and for the copulation. An experiment was terminated after thirty minutes without any interaction. Observation sessions were done in parallel.

In a second series of experiments we investigated the extent of occurrence of the different interspecific interactions observed in the first series. First we examined the number of interspecific interactions between a *gibbosus* male and an *O. fuscus* male in the presence of an *O. gibbosus* female. We also did this in the absence of an *O. gibbosus* female. Next we investigated the number of interactions between a *gibbosus* male and an *O. fuscus* female in the absence of an *O. gibbosus* female. Each time we also did analogous experiments with *tuberosus* males. Finally we observed the number of interactions between two *gibbosus* males (in either the absence or presence of an *O. gibbosus* female), between two *tuberosus* males and two *O. fuscus* males (in the absence of an *O. gibbosus* female). For each experiment we observed 10 repeats in parallel during five hours. The *gibbosus*/*tuberosus* male as well as *Oedothorax fuscus* spider(s) were again put in the vial of the *O. gibbosus* female; in the absence of the last we put *O. fuscus* spiders in the vial of the *gibbosus*/*tuberosus* spider.

In a third series of experiments we investigated whether there are interactions between *gibbosus* and *tuberosus*. Each time we put one *gibbosus* male and one *tuberosus* male at the same time in the vial of an *O. gibbosus* female. With this experimental design we should be able to test whether *gibbosus* is sexually more attractive than *tuberosus*. Does the female choose more often to copulate with *gibbosus* than *tuberosus*? Is the copulation of *gibbosus* longer than this of *tuberosus*? In a first experiment with 59 repeats we stopped observations if nothing happened after 30 minutes; in a second set-up (9 repeats) we observed during a whole day.

The following statistical tests were used : one-way-ANOVA, Fisher-test and Chi-square test.

RESULTS

Interspecific homo- and heterosexual interactions between *O. gibbosus* and *O. fuscus*

In the first experiment a *gibbosus* morph male and an *O. fuscus* female were placed in the vial of an *O. gibbosus* female. The *O. fuscus* female grasped the *O. gibbosus* male, appearing to feed from the cephalic groove for 7 minutes. During this time the *O. gibbosus* male tried to copulate but did not succeed.

TABLE 1

First experiment of interspecific homo- and heterosexual interactions between the *gibbosus* male morph (*O. gibbosus*) and spider(s) of *O. fuscus*, in the cup of the *O. gibbosus* female. The duration of these interactions are included in the table. In the 5th part of the experiment we added one *gibbosus* male and seven *O. fuscus* males; in the 6th part of the experiment we added two *gibbosus* males.

nr	female	added morph of *O. gibbosus*	added spiders of *O. fuscus*	gustatorial courtship between	duration
1	*O. gibbosus* female	*gibbosus* male	1 *O. fuscus* female	*gibbosus* male & *O. fuscus* female	7 min
2	*O. gibbosus* female	*gibbosus* male	1 *O. fuscus* male	*gibbosus* male & *O. gibbosus* female	10 min
				gibbosus male & *O. fuscus* male	2 min
				gibbosus male & *O. fuscus* male	3 sec
				gibbosus male & *O. fuscus* male	2 sec
3	*O. gibbosus* female	*gibbosus* male	1 *O. fuscus* male	*gibbosus* male & *O. fuscus* male	5 min
				gibbosus male & *O. fuscus* male	2 min
				gibbosus male & *O. fuscus* male	6 sec
4	*O. gibbosus* female	*gibbosus* male	1 *O. fuscus* male	*gibbosus* male & *O. fuscus* male	6 min
				gibbosus male & *O. fuscus* male	4 min
5	*O. gibbosus* female	*gibbosus* male	7 *O. fuscus* males	*gibbosus* male & *O. fuscus* male	5 min
				gibbosus male & *O. fuscus* male	1 min
				gibbosus male & *O. fuscus* male	5 sec
				gibbosus male & *O. fuscus* male	30 sec
				gibbosus male & *O. fuscus* male	5 min
				gibbosus male & *O. fuscus* male	1 min
				gibbosus male & *O. fuscus* male	6 min
				gibbosus male & *O. fuscus* male	1 min
nr	**female**	**added morph of *O. gibbosus***	**added spiders of *O. gibbosus***	**gustatorial courtship between**	**duration**
6	*O. gibbosus* female	*gibbosus* male	1 *gibbosus* male	*gibbosus* male & *gibbosus* male	12 min

TABLE 2

Second experiment of interspecific homo- and heterosexual interactions between the *tuberosus* male morph (*O. gibbosus*) and spider(s) of *O. fuscus*, in the cup of the *O. gibbosus* female. The duration of the observed intraspecific copulations are included in the table and there were not any interspecific interactions in this experiment.

nr	female	added morph of *O. gibbosus*	added spiders of *O. fuscus*	courtship and copulation between	copulation time
1	*O. gibbosus* female	*tuberosus* male	1 *O. fuscus* female	*tuberosus* male & *O. gibbosus* female	70 min
2	*O. gibbosus* female	*tuberosus* male	1 *O. fuscus* male	*tuberosus* male & *O. gibbosus* female	67 min
				tuberosus male & *O. gibbosus* female	64 min
3	*O. gibbosus* female	*tuberosus* male	1 *O. fuscus* male	*tuberosus* male & *O. gibbosus* female	67 min
4	*O. gibbosus* female	*tuberosus* male	1 *O. fuscus* male	*tuberosus* male & *O. gibbosus* female	66 min
5	*O. gibbosus* female	*tuberosus* male	1 *O. fuscus* male	*tuberosus* male & *O. gibbosus* female	68 min
6	*O. gibbosus* female	*tuberosus* male	1 *O. fuscus* male	*tuberosus* male & *O. gibbosus* female	70 min

Is such contact also possible between an *O. fuscus* male and a *gibbosus* male morph? In a second experiment a *gibbosus* morph male and an *O. fuscus* male were therefore brought together with an *O. gibbosus* female. Initially, the *gibbosus* male and *O. gibbosus* female engaged in a copulation posture for 10 minutes, but without palp insertion. Shortly after the copulatory posture ended the *O. fuscus* male grasped the *gibbosus* male at its cephalic groove for 2 minutes (Fig. 2). The *O. fuscus* male showed feeding behaviour during the whole time interval, while the *O. gibbosus* male drummed its palps on the ventral side of the *O. fuscus* male. The same interspecific behaviour was repeated briefly two times. A third and a fourth experiment were analogous to the second experiment and resulted in respectively three (5 min, 2 min and 6 sec) and two interspecific interactions (6 min and 4 min) between an *O. fuscus* male and a *gibbosus* male morph.

Fig. 2. – Homosexual interaction between a *Oedothorax fuscus* male (top) and a *gibbosus* male morph. The *O. fuscus* male has grasped the *gibbosus* male at its cephalic groove and shows feeding behaviour.

In a fifth experiment seven *O. fuscus* males were combined with one *O. gibbosus* female and one *gibbosus* morph male. We used seven males to make it more difficult for the *O. gibbosus* female to choose. Several *O. fuscus* males displayed gustatorial courtship postures; altogether there were eight interspecific homosexual courtships. During most interactions the haemotodoch of the male palp was already visible.

In a sixth experiment two *gibbosus* males were brought together with an *O. gibbosus* female to observe if a gustatorial courtship is also possible between two *gibbosus* morph males. There was no contest between the two *gibbosus* morph males for the *O. gibbosus* female; instead of this, a gustatorial interaction happened between the two *gibbosus* male morphs during 12 minutes.

We also did analogous experiments with *tuberosus* males; but there was no resultant contact between the *tuberosus* morph male and the *O. fuscus* males or females. In the experiment with a *tuberosus* male, an *O. gibbosus* female and an *O. fuscus* female there occurred one intraspecific copulation (70 min). In one of the five experiments with a *tuberosus* male, an *O. gibbosus* female and an *O. fuscus* male there occurred two intraspecific copulations (67 min and 64 min respectively) and in each of the others there was only one intraspecific copulation (67, 66, 68 and 70 min respectively).

Because of the aforementioned interactions between the *gibbosus* morph male and the both sexes of *O. fuscus*, it was impossible to compare the number of copulations respectively achieved by *gibbosus* and *tuberosus.*

The extent of interspecific interactions between *O. gibbosus* and *O. fuscus*

In the experimental series with a *gibbosus* male and an *O. fuscus* male in presence of an *O. gibbosus* female, interspecific courtships between a *gibbosus* male and an *O. fuscus* male occurred in seven of the ten cases (in one case twice, in another three times and in the others once). *Tuberosus* did, however, also perform in five of the ten repeats an intraspecific copulation; *gibbosus* only did so in three cases. In the absence of an *O. gibbosus* female it only occurred in three of the ten repeats (each time twice). In the analogous experiments with the *tuberosus* male there occurred no interspecific courtship between *tuberosus* and an *O. fuscus* male, either in the presence or absence of an *O. gibbosus* female.

In the experiment with a *gibbosus* male and an *O. fuscus* female, interspecific courtship occurred in only two of the ten repeats (in one case once, in the other twice). In the combination of a *tuberosus* male and an *O. fuscus* female, in contrast with the first experiment, in five of the ten repeats interspecific courtships occurred (in one case twice, in another three times and in the others one).

Finally, in the ten repeats of two *gibbosus* males no gustatorial interactions were observed, in contrast with the first experiment, in either the presence or the absence of an *O. gibbosus* female; the same was seen in the ten repeats of two *tuberosus* males and two *O. fuscus* males.

Is there a gustatorial interaction between *gibbosus* and *tuberosus* males?

In the 68 (59+9) cases of an *O. gibbosus* female to which a *gibbosus* and a *tuberosus* male were added we never observed a gustatorial courtship between *gibbosus* and *tuberosus* males. The only kinds of interactions between both male morphs that occurred were aggressive contacts or disturbance of copulation. *Tuberosus* sometimes also disturbed the gustatorial courtship between *gibbosus* and the female. This disturbance of the other male morph only exceptionally led to an interruption of the copulation or gustatorial courtship.

In the first experiment there was no significant difference in the number of allowed copulations between the two male morphs according to a Fisher test ($p = 0{,}1861$); the female chose nine times (on 50 refusals) for *gibbosus* and 15 times (on 44 refusals) for *tuberosus*. If the observation time was prolonged to a whole day, the number of allowed copulations between both male morphs was also not significantly different. The female chose only three times (on six refusals) for *gibbosus* and four times (on five refusals) for *tuberosus.*

Does *tuberosus* copulate longer than *gibbosus*? According to different ANOVA tests on the data of the first part of the experiment, there was no significant difference in duration of the first palp insertion between both male morphs (ANOVA : df Effect = 1, df Error = 6, $F = 0{,}0938$; $p = 0{,}770$; $\bar{x}_{gib} = 35 \pm 4{,}58$ min; $n = 9$; $\bar{x}_{tub} = 33{,}87 \pm 4{,}31$ min; $n = 15$), either in duration of the second palp insertion (ANOVA : df Effect = 1, df Error = 6, $F = 0{,}662$; $p = 0{,}447$; $\bar{x}_{gib} = 34{,}83 \pm 6{,}55$ min; $n = 6$; $\bar{x}_{tub} = 30{,}5 \pm 6{,}36$ min; $n = 2$), or in the duration of the complete copulation (both palps) (ANOVA : df Effect = 1, df Error = 6, $F = 0{,}0611$; $p = 0{,}464$; $\bar{x}_{gib} = 41{,}78 \pm 13{,}20$ min; $n = 9$; $\bar{x}_{tub} = 47{,}83 \pm 18{,}87$ min; $n = 15$). In the experiment with the long observation period there were only enough results to examine the difference in duration of the first palp insertion. No significant difference for the first palp insertion between both male morphs was found (ANOVA : df Effect = 1, df Error = 5, $F = 0{,}973$; $p = 0{,}369$; $\bar{x}_{gib} = 36{,}33 \pm 9{,}86$ min; $n = 3$; $\bar{x}_{tub} = 27{,}5 \pm 12{,}82$ min; $n = 4$).

DISCUSSION

According to the lock-and-key hypothesis genitalia and non-genitalic contact structures evolve by pre-insemination hybridisation avoidance (Arnqvist, 1998). According to several publications the lock-and-key hypothesis has fared poorly in many attempts to test it (Shapiro & Porter, 1989; Arnqvist et al., 1997; Arnqvist & Thornhill, 1998).

Schaible et al. (1986) suggested that the most important function of the male head structures of the *gibbosus* morph male is the fixation of the position for copulation. This could be in agreement with the lock-and-key hypothesis. This is, however, quite improbable because the contact between the female chelicerae and male head structures in most cases does not last during the copulation. Also the observed homo- and heterosexual interspecific contacts between males or females of *O. fuscus* and

the *gibbosus* male are in disagreement with the lock-and-key hypothesis. These interspecific gustatorial courtships are useless sexual interactions and the carapace structure of the *gibbosus* male cannot, therefore, be considered to have evolved in order to avoid interspecific sexual interactions. We also observed interspecific gustatorial courtships between an *O. retusus* (Westring, 1851) male and a *gibbosus* male (Vanacker, unpubl. data). *Gibbosus* even tried to copulate with *O. fuscus* males, which is evidently also a waste of energy. Not being occupied with such kinds of interspecific gustatorial courtships, *tuberosus* has more opportunity to copulate with an *O. gibbosus* female. In the first series of experiments *gibbosus* was indeed only able to perform one intraspecific gustatorial courtship not leading to a copulation. We never observed *tuberosus* males making use of the hairy groove of *gibbosus*, but two *gibbosus* males on the other hand can perform a gustatorial courtship. Presumably, this last interaction occurs only rarely; in the second experiment we did not observe it at all.

Besides pheromones, the hunch of *gibbosus* probably also secretes a nuptial gift because this hunch is completely filled with gland cells of different kinds (Vanacker, unpubl. data). Nuptial feeding is already known to occur in some spiders, such as the red back spider (Forster, 1992; Andrade, 1996) and *Pisaura mirabilis* (Clerck, 1757) (Lang, 1996; Nitzsche, 1999; Stalhandske, 2001). If we assume that *gibbosus* indeed produces a nuptial gift, *O. fuscus* males and females could be attracted by the smell of the nutritional fluid itself, by male pheromones or by a combination of both. These interspecific "gustatorial courtships" can probably be interpreted as "robbery" of the nuptial gift at the cost of the *gibbosus* males; this could also explain the '"gustorial courtship" between the two *gibbosus* males. It is surprising, however, that *tuberosus* does not rob the nuptial gift of its "competitor" *gibbosus*. In the field these interspecific interactions might not be a real problem because of the aggregated distribution of *O. gibbosus* spiders in the moss. The only interspecific interaction that *tuberosus* males perform is with an *O. fuscus* female, suggesting poorly developed reproductive isolation between both species. It is striking that more interspecific interactions occur in the presence of an *O. gibbosus* female; the chance that *gibbosus* males make a wrong choice seems to be greater if they are excited by female pheromones.

Also the "sperm holder hypothesis" (see above) cannot hold for the cephalic structures of *Oedothorax gibbosus*; this hypothesis is specific for pholcid spiders. Sexual selection by female choice or sexual conflict is thus probably the force for the evolution of the cephalic structures of male erigonines.

Is *gibbosus* now sexually more attractive than *tuberosus*? We cannot answer this question yet on the basis of the third experiment. The presence of both male morphs in the vial of the female probably provokes too much disturbance; comparing *gibbosus* male – *Oedothorax gibbosus* female and *tuberosus* male – *Oedothorax gibbosus* female couples separately could solve this problem.

The results we obtained thus far, however, show that dwarf spiders probably are excellent candidates for speciation research. Besides further species-recognition experiments between different *Oedothorax* species, we will study the interactions between different *Diplocephalus* Bertkau, 1883 species in the near future. Also more genetic and histological research is necessary.

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Cloning and tissue distribution of the cyclic AMP generating peptide of the grey flesh fly *Neobellieria bullata* (Diptera : Sarcophagidae)

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ABSTRACT. Originally, two forms of cyclic AMP Generating Peptide (cGP) were identified in whole body extracts of the flesh fly *Neobellieria bullata*. The long form, a 48-mer, stimulated cAMP production by Malpighian tubules of *Manduca sexta*. The short form, which had been discovered previously with an ovarian bioassay, turned out to be the 1-15 aa sequence of the 48-mer. We here report on the cloning, sequencing and expression profile of the cGP-gene in *N. bullata*. The full-length cDNA sequence, obtained by RT-PCR in combination with 5´ and 3´ RACE, encodes a single copy of the cGP precursor. No signal peptide is present in this precursor, which, compared to the mature peptide, is only extended at the N-terminus with an extra methionine residue. RT-PCR revealed that Neb-cGP is expressed in both larval and adult brain, testis and ovaries, flight muscles, Malpighian tubules, midgut, and fat body.

KEY WORDS : insect endocrinology, reproduction, neuropeptide, gonadotropin, testis.

INTRODUCTION

In an attempt to identify new hypothalamic hormones regulating anterior pituitary hormone secretion, MIYATA & collaborators (1989) discovered a novel member of the secretin/glucagon/VIP peptide family. This peptide was called Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), reflecting its ability to potently stimulate cAMP accumulation in cultured adenohypophyseal cells. PACAP occurs as two variants, PACAP38 (a 38 amino-acid polypeptide) and the C-terminally truncated form PACAP27. The evolutionary origin and history of PACAP in Chordates, from Tunicates to mammals and birds, are well documented (VAUDRY et al., 2000).

In line with the PACAP research in vertebrates, SCHOOFS et al. (1994) engaged in the search for cAMP-stimulating peptides in insects, in particular in the flesh fly *Neobellieria* (=*Sarcophaga) bullata* Parker, 1916. First, a 15-mer peptide was isolated that potently stimulated cAMP production by young vitellogenic ovaries. Next, a 48-mer was isolated by using by Malpighian tubules of *Manduca sexta* Linnaeus, 1763 (SPITTAELS et al., 1996) as a bioassay. It turned out that the 15-mer peptide is the N-terminal sequence of the 48-mer. Neither the 15-mer nor the 48-mer displays any substantial structural similarity to PACAP. However, a ubiquitous 58-mer protein displaying pronounced sequence similarity with cGP has recently been identified in rats, treated with Streptozotocin, a glucose analogue that induces type-1 diabetes by destroying the pancreatic β-cells. This protein was named DAPIT (diabetes-1 associated protein in insulin sensitive tissues) (PAIVARINNE & KAINULAINEN, 2001). Genes coding for analogue proteins have been described in several vertebrate species, but in all of them their function remains enigmatic.

In this paper we report the cloning and the expression pattern of the 48-mer cGP gene and its similarity to the as yet functionally uncharacterized *Drosophila* gene CG15304. In addition we also measured the effect of both the 1-15 mer and full-length fly cGP on cAMP production by several fly tissues.

MATERIAL AND METHODS

Breeding and tissue collection

Neobellieria bullata individuals were reared as described by HUYBRECHTS & DE LOOF (1982). Staging of ovarian development was done according to PAPPAS & FRAENKEL (1978) BYLEMANS et al. (1997). Ovaries are staged as 4A, 4B, 4C and M when the terminal oocytes occupy 25, 50, 75 and 100%, respectively, of the total length of the ovarian follicle. As long as the larvae feed on liver, they are referred to as liver-stage larvae (LsL). After they stop feeding they start searching for a dry environment [wandering stage (ws)] and finally pupate 3 days later.

Female flies in the stages of 4B and 4C and males of corresponding age were collected, lightly anaesthetized with CO_2 and dissected in *Neobellieria* Ringer (121.5 mM NaCl, 10 mM KCl, 1.0 mM NaH_2PO_4, 10 mM $NaHCO_3$, 0.7 mM $MgCl_2$, 2.2 mM $CaCl_2$, pH 6.8 (BYLEMANS et al., 1998)).

To study the expression of Neb-cGP at the level of mRNA, the following tissues were collected and immediately transferred to liquid nitrogen. From adults of both sexes : batches of 20 gonads, 100 Malpighian tubules, 20 midguts, 20 abdominal fat body masses and 10 flight muscle masses. From larvae (unsexed) in the wandering stage : 100 brains.

For cAMP measurements, the following individual organs were dissected and transferred to the wells of a micro-titre plate containing 100 µl of IBMX (100 µM) in *Neobellieria* Ringer : ovary (4A and 4B stage), testis, brain, a piece of flight muscle, fat body and Malphigian tubule.

RT-PCR, Cloning and Sequencing

mRNA was purified from brains of vitellogenic females using the Quick prep® micro mRNA purification kit (Amersham, Pharmacia Biotech) according to the manufacturer's instructions. Single stranded cDNA was synthesized according to the protocol of the Marathon™ cDNA amplification Kit (Clontech Laboratories Inc) : 1 µg of mRNA was reverse transcribed using 20 units of AMV reverse transcriptase and the provided cDNA synthesis primer.

Based on the known sequence of Neb-cGP 48–mer (Spittaels et al., 1996) two degenerated primers, spanning a theoretical cGP cDNA internal fragment of 120 bp, were designed and obtained from Eurogentec, Belgium.

1. cAMPGRP F :
   5′GCNGARAARYTNWSNGGNYTN3′
2. cAMPGRP R :
   5′GGYTTCATNACRTTRTANGCDA3′

PCR reaction mixtures contained 2 µl (<0.1µg) of brain cDNA template, 1 µl of 50X Advantage® 2 Polymerase Mix, 5 µl of 10X Advantage® 2 PCR Buffer, 1 µl of each dNTPs (10 mM), 5 µl of each primers (10 µM) and H_2O added up to a 50 µl reaction volume. Hot start PCR was performed in a Thermotrio-Block TB-1 Thermocycler (Biometra). Thermal cycling consisted of 30 cycles with a denaturation for 1 min at 94˚C, annealing step for 1 min at 35˚C, extension for 1 min at 68˚C and an extra elongation step for 5 min at 68˚C. A second PCR amplification, using 1 µl of the above PCR result and identical PCR conditions was carried out.

For cloning, the PCR products of the second round were first electrophoretically separated in a 3.5% Nusieve® GTG® (low melting) agarose (Sanvertech) gel in 1X TAE buffer. The band of the expected size was excised and directly ligated into PCR® 2.1-Topo vector (low melt agarose method). This ligation product was used to transform competent *E. coli* Lacz(cells according to the protocol of the Topo TA cloning ® Kit (version I, Invitrogen, The Netherlands).

Recombinant plasmid DNA was isolated from the obtained white colonies according to the instructions of the manufacturer of the High pure plasmid Isolation Kit (Roche Molecular Biochemicals, Germany). Automated sequencing was performed on an ABI Prism(R) sequencer 310 (Applied Biosystems) using the ABI PRISM(R) Big Dye™ Terminator Cycle sequencing Ready Reaction Kit version 2.0 (PE Biosynthesis).

Rapid amplification of cDNA ends and identification of full length nucleotide sequence of Neb-cGP

In order to obtain a complete nucleotide sequence, Rapid Amplification of cDNA Ends (RACE) was employed (Frohman et al., 1998; Janssen et al., 2001). 5′ and 3′ ends RACE PCR, and 3′-nested RACE PCR was performed according to the protocol of the Marathon ™ cDNA amplification kit (Clontech, Westburg, The Netherlands). Adaptor-ligated double stranded cDNA served as template for these RACE PCR. Single stranded cDNA (as described previously) was now synthesized from total RNA that was isolated from 100 mg of brain tissue of young vitellogenic females using Trizol Reagent (GIBCO-BRL) (Chomczynski & Saachi, 1987).

5′ and 3′ RACE reactions were performed using Marathon Adaptor Primer (AP_1, Clontech) in combination with one of the respective gene specific primers, which were constructed according to the previously obtained sequence information : 5′-ATG-CGA-TGA-TCA-AAC-CAA-TG-3′ for 5' RACE and 5′- CAT-GGC-TGG-ACG-TGC-TAA-TGT-TGC-3′ for 3' RACE.

In the case of 3′ end RACE reaction, nested PCR were performed using nested AP_2 primer (Clontech) and nested gene specific primer 5′CATCGCATACAACGTAATGAAGCC3′. RACE products were analysed by 1.2% agarose gel electrophoresis and purified using Qiaex II DNA extraction kit (Qiagen). Purified DNA fragments were cloned into PCR® 2.1 Lacz α vector employing the TOPO TA Cloning kit (Invitrogen). Further downstream processing, up to sequencing of the inserts, was done as described above.

Sequence analysis

Blast programs were used to search databases for sequence homologies (Altschul et al., 1997). Multiple sequence alignment results were obtained by employing the Clustal W program (European Bioinformatics Institute). For prediction of the presence of a signal peptide, the sequence was analysed with Prot param tool and Signal P V2.O web site.

RT-PCR : Tissue specific expression profile

The expression of Neb-cGP in different organs/tissues of both sexes (brain, flight muscle, midgut, Malpighian tubule, fat body, testes and ovaries) was examined using reverse transcriptase PCR (Peeters et al., 1999). Single stranded cDNAs were prepared using total RNA as described before. Neb-cGP specific primers (5′CGGTGCTGAAGCT-GAAAAAT-3′, 5′GGCAATGATCAAACCATGA-3′, Eurogentec, Belgium) were used for the subsequent PCR. Hot start PCR was performed for 30 cycles with a denaturation step for 1 min at 94˚C, annealing for 1 min at 45˚C and elongation for 30 sec at 68˚C. As a positive control for cDNA quality actin primers were used. The amplified PCR products were visualized under UV transillumination following 1.5% agarose gel electrophoresis.

cAMP stimulation assay

Synthetic 1-15 mer (Invitrogen) and full-length peptide (a kind gift from Dr. Hui, Tian, Tularik, San Francisco) of Neb-cGP were tested for their ability to stimulate cAMP production in the different organs/tissues of *Neobellieria*.

Following dissection, the organs/tissues were incubated in wells of a microtitre plate for 1h at 35˚C in 100 µl of Ringer containing 100 µM IBMX. After 1h, 25 µl of the incubation medium was removed for quantification of basal release level of cAMP and replaced by 25 µl of test peptide dissolved in IBMX-Ringer. Incubation at 35˚C continued for 1h. Next, a 25 µl aliquot of incubation medium was taken from each well for cAMP measurement according to the manufacturers instructions (Amersham ^{3}H cAMP assay kit).

RESULTS

Cloning of full–length Neb-cGP cDNA sequence

According to the information provided in the Materials and Methods section, two subsequent PCR reactions at a rather low annealing temperature of 35˚C and using a degenerated primer combination were needed for obtaining an internal Neb-cGP cDNA fragment starting from brain mRNA. The resulting fragment provided sequence information for designing the gene specific primers used in the subsequent RACE reactions. Combination of the sequence information obtained with 5′ and 3′ RACE allowed the reconstitution of the full-length cDNA sequence (Fig. 1) (GenBank accession number AY141181). *In silico* translation of the cDNA revealed a singly open reading frame corresponding to the Neb-cGP precursor, which lacks a signal peptide and, when compared to the purified mature peptide, only has an extra N-terminal methionine. Although no conserved polyadenylation motif can be traced in the cDNA sequence, a 3′ poly A stretch is clearly present in addition to a short 5′ leader and 3′ trailer sequence. The entire cDNA sequence up to the poly A tail is only 188 bp long.

```
                  M  A  G  A  E  A  E  K  L  S  K  L  S  K  Y
5′- taataacaaca**ATG**GCCGGTGCTGAAGCTGAAAAATTAAGTGGTTTATCCAAATAT
 F  N  G  T  T  M  A  G  R  A  N  V  A  K  A  T  Y  A  V  I
TTCAATGGTACAACCATGGCTGGACGTGCTAATGTTGCTAAAGCCACTTATGCTGTCATTG
 G L  I  I  A  Y  N  V  M  K  P  K  K  K
GTTTGATCATTGCCTACAATGTCATGAAACCCAAAAAAAAAG**taa**attttgttattgttgtt
acaattcgca*AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA*-3′
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Fig. 1. – Complete cDNA sequence with deduced amino acid sequence of *N. bullata* cyclic AMP Generating Peptide. Start (ATG) and stop (taa) codon marked in bold, Poly-A tail shown in italics.

Blast searches for conserved genes in other species pointed towards the occurrence of an homologous gene (AE003450, CG15304) in *Drosophila* showing 67% sequence identity at cDNA level (Fig. 2A) and 81% at the amino acid level. Both Neb-cGP and the corresponding *Drosophila* cDNA share 46% identity with *Rattus norvegicus* DAPIT cDNA. At the amino acid sequence level (Fig.2B) Neb-cGP shows 43% identity and 62% similarity to this ubiquitously expressed 58-mer DAPIT protein originally found in diabetic rats. There are homologs of DAPIT in all studied mammals as well as in *Xenopus* (Paivarinne & Kainulainen, 2001).

RT-PCR

Tissue specific expression of Neb-cGP in a variety of tissues was examined by RT-PCR (Fig. 3 A and B). The cDNA quality of all samples was confirmed by generating an actin fragment (data not shown). A large amount of mRNA encoding Neb-cGP was detected in all tissues tested, namely brain, flight muscle, midgut, Malpighian tubules, fat body, testis and 4A ovaries. Not only the adult brain but the larval one as well expresses Neb-cGP gene.

cAMP stimulation by Neb-cGP

Because only small amounts of the synthetic full-length 48 mer fly peptide were available, only a few experiments with ovaries and testis but not with other tissues could be done. The 1-15 mer peptide of Neb-cGP could be tested more extensively, namely on 4A and 4B stage ovaries, testis, flight muscle Malphigian tubules, brain and fat body. In all tissues tested the basic rate of cAMP release into the incubation medium during 1 h incubation varied from 0.7 to 1.4 (±0.1-0.6 SD) pmol. Substantial stimulation of cAMP release could only be obtained in testis and 4A ovaries. The strongest stimulation, namely sevenfold, was obtained for testis with the 1-15 mer peptide at a final concentration of 17.5 µM (9.8 (3.3 pmol/h versus 1.4 (0.6 pmol/h in the control). At the same dose the 1-15 mer less potently stimulated the ovaries (1.7±0.4 pmol/h versus 1.3 ±0.1 pmol in the control). Three replications were used for each dose.

TABLE 1

Activity of 1-15 mer cGP in different tissues (data in parenthesis indicate only single replication for 1-48 mer peptide)

Tissues	Pre incubation Mean ± SD (Picomol/h)	After incubation Mean ± SD (Picomol/h)
Testis	1.40 ± 0.58 (0.59)	9.84 ± 3.26 (15.37)
Ovary	1.32 ± 0.08 (0.71)	1.70 ± 0.40 (3.64)
Flight muscle	1.08 ± 0.03	1.28 ± 0.05
Malphigian tubules	0.91± 0.03	1.04 ± 0.04
Fat body	1.40 ± 0.07	1.39 ± 0.23
Brain	0.7 ± 0.05	0.7 ± 0.01

At a concentration of 27 µM full-length peptide provoked a very strong cAMP production in testis (15.4 pmol/h versus 0.6 pmol/h in the control). Ovaries were less stimulated than testis (3.6 pmol/h versus 0.7 pmol/ h in the control at the same concentration).

```
Neobellieria    ------------------------------------------------------------
Drosophila      ATCGAAGACCGATAGACCTCGATAGACTCAGTTGTATAACCTCTGCTCGCAAATATTTTT 60
Rattus          ------------------------------------------------------------

Neobellieria    ------------------------------TAATAA--CAACAATGGCCGGTGCTGAAGC 28
Drosophila      TACAGGCAACTAGCAGCTCGAATTCCGTGCAAATAAACCTTTAAAAATGGCTGGCGAAGG 120
Rattus          -----GTGATCGGACGAAGAAGATTGAAGTCATGGCTGGCCCAGAAAGTGATGGCCAATT 55
                                          *          *      * **   **

Neobellieria    TGAAAAATTAAGTGGTTTATCCAAATATTTCAATGGTACAACCATGGCTGGACGTGCTAA 88
Drosophila      CGAGAAACTGACCGGCCTGTCGAAAATCTTCAATGGCACCACCATGAGCGGCCGTGCAAA 180
Rattus          CCAG---TTCACTGGTATTAAAAAATATTTCAACTCTTATACCCTCACAGGTAGAATGAA 112
                  *     * *  **  *    ***   *****       *** *    **  *    **

Neobellieria    TGTTGCTAAAGCCACTTATGCTGTCATTGGTTTGATCATTGCCTACAATGTCATGAAACC 148
Dosophila       TGTGGCCAAGGCCACGTACGCCGTGATGGGCCTGCTGATCGCCTACCAGGTGCTGAAGCC 240
Rattus          TTGTGTCCTGGCCACATATGGAGGCATTGCTTTGTTGGTCCTATACTTTAAGTTAAGGCC 172
                *   *     ***** ** *  *  ** *   ** *  *    ***       * *  **

Neobellieria    CAAAAAAAAGTA---------AATTTTGTTATTGTTGTTACAATTCGCAAAAAAAAAAAA 199
Drosophila      CAAGAAGAAGTAGAGGGATGCAACTGCTCCAACGCGGCTACAACTCATCCGCCACAATCG 300
Rattus          TAAAAAAACCCCAGCTGTGAAAGCAACATAAATGGATTTTGAAATGTCTGGCCTTATCTG 232
                 ** ** *             *        *  *    *  ** *          *

Neobellieria    --AAAAAAAAAAAAAAAAAA---------------------------------------- 218
Drosophila      --ATGTCTTGGATCCGGGGCCATCGCTG-GACTGCGGATCAGTTTATTTTATGTGTAGTG 357
Rattus          TTAAGTCCCACGCCTGAAGAAGCTGATGTGAACTCATCATGTAATACTCAATTTGTACAA 292
                  *

Neobellieria    ------------------------------------------------------------
Drosophila      CAAGACCTTGAAGGAATAAATAAGCTGAGAATGTAACACACTGCAAAAAAAAAAAAAAAA 417
Rattus          TAA-ATTATGAACCTGGAAAAAAAAAAAAAAA---------------------------- 322
```

Fig. 2. – Nucleotide and protein sequence alignment.

2A. Nucleotide sequence alignment-showing comparison between Neb-cGP, the *Drosophila* homolog and the *Rattus norvegicus* DAPIT. Residues that are identical in all precursors are indicated with an asterisk (*).

```
Neobellieria      -AGAEAE---KLSGLSKYFNGTTMAGRANVAKATYAVIGLIIAYNVMKPKKK------ 48
Drosophila        -MAGEGE---KLTGLSKIFNGTTMSGRANVAKATYAVMGLLIAYQVLKPKKK------ 48
Rattus            MAGPESDGQFQFTGIKKYFNSYTLTGRMNCVLATYGGIALLVLYFKLRPKKTPAVKAT 58
                    . *.:   :::*:.* **. *::** * . ***. :.*:: *  ::***.
```

2B. Sequence alignment for the cGP protein of *Neobellieria*, *Drosophila,* and *Rattus norvegicus* DAPIT. Symbols under each set of lines indicate identity (*), highly conserved substitutions (:) and conserved substitutions (.).

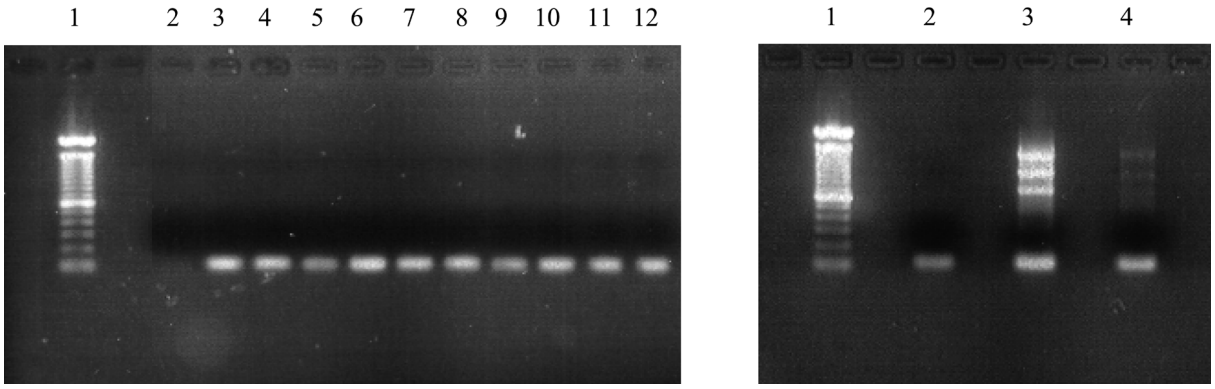

Fig. 3. – Tissue distribution of Neb-cGP as revealed by RT-PCR.

3A (left). Lane 1 : 100 bp DNA ladder. Lane 2 : Water control, Lane 3 and 4 : Flight muscle from female and male flies. Lane 5 and 6 : Midgut from female and male flies. Lane 7 and 8 : Malpighian tubules from female and male flies. Lane 9 and 10 : Fat bodies from female and male flies. Lane 11 and 12 : ovary and testis.

3B (right). Lane 1 : DNA size marker. Lane 2 and 3 : Brain from female and male flies. Lane 4 : Larval brain.

DISCUSSION

The aa-sequence of 48-mer cGP as originally reported by SPITTAELS et al. (1996) was confirmed by our sequencing of the cGP cDNA with its single open reading frame. The 1-15 mer is probably a proteolytic cleavage product of the 48-mer but the aa sequence at position 15-16, namely phenylalanine-asparagine, is not a common cleavage site. In a mass spectrometrical analysis of the corpus cardiacum of *Neobellieria*, BAGGERMAN et al. (2002) could only detect the 48-mer, not the 1-15 mer. Obviously the 1-15 mer was a degradation product that was formed during the extraction procedure as applied by SCHOOFS et al. (1994). Using immunocytochemistry these authors identified cGP immunopositive cells in the pars lateralis of the brain but unfortunately they did not test other tissues. In combination with their mass spectrometric detection of the 48-mer in extracts of fleshfly corpora cardiaca they suggested synthesis of cGP in the brain followed by storage in and subsequent release from the corpora cardiaca. Accordingly cGP is assumed to be a neurohormone. The results obtained in the bio-assay, using distinct tissues and major response to exogenously applied cGP being observed with testis and to a minor extend with ovaries as well, make it tempting to speculate about cGP being a true gonadotropin.

Our cDNA sequencing data are in conflict with this assumption. The usual situation is that the precursor of secreted proteins contains a signal peptide that is cleaved off when the peptide is secreted. However, there is no signal peptide encoded in the cGP cDNA, neither in *Neobellieria*, nor in *Drosophila*. Until the opposite will be proven we have to assume that the mature cGP peptide is not released from the cells where it is produced. Anyhow, in receptive tissues there should be a signalling system that is activated by exogenously applied cGP. Either cGP interacts with an as yet unidentified plasma membrane receptor or the peptide can surpass the plasma membrane and directly interact with the cAMP generating pathway intracellularly. This last possibility has been suggested for the related DAPIT protein by PAIVARINNE AND KAINULAINEN (2001). They used several software programmes such as HMMTOP, TMpred, DAS and SPLIT to obtain conformational information about DAPIT. Their results suggest the possibility of an outside-inside oriented transmembrane helix at the C-terminus while the N-terminus should be directed outwards in both DAPIT protein and Neb-cGP. If this prediction is correct, direct signal transduction activity of this externally applied protein could be realistic. On the other hand our own observation that the 1-15-mer N-terminal sequence suffices for stimulating cAMP production does not support this assumption.

The wide tissue distribution of cGP in flies, and the occurrence of similar proteins throughout the animal Kingdom where the coding mRNA also have such an ubiquitous tissue distribution, suggest that this family of proteins somehow plays a key role in some physiological processes. It will be a challenge to uncover its exact role(s).

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Diet composition and prey choice by the red-backed shrike *Lanius collurio* in western Poland

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ABSTRACT. We investigated diet and prey choice in a population of red-backed shrike (*Lanius collurio*) living in an intensively used farmland (W Poland). Diet was estimated by three methods : collars in nestlings, and pellets and prey remains in larders. Insects, mainly Coleoptera, Hymenoptera and Orthoptera constituted 97.7% of the diet, with a total of 4392 prey items identified from all samples. However, during rainy and cold days vertebrates formed an important component (up to 26.5% by biomass) of the food of the red-backed shrike.

Food preference – expressed in relation to availability – was estimated for five arthropod taxa. Hymentoptera, Orthoptera and Coleoptera were more preferred prey. Heteroptera and "other invertebrates", included mainly flies, dragonflies and spiders were less preferred.

For rational management of the red-backed shrike in farmland, we suggest that places with available prey (in appropriate densities), small vegetation patches and perches suited to low-expenditure hunting strategy, should be preserved, as well as established.

KEY WORDS : diet, food selection, insects, vertebrates, *Lanius collurio.*

INTRODUCTION

The red-backed shrike *Lanius collurio* (Linnaeus, 1758) is the most numerous of the shrike species to breed in the Western Palaearctic. However, decline of the local populations over many areas within the geographical range of the species has been primarily attributed to a loss of suitable habitat. Changes in agricultural practices were thought to be partly responsible for a reduction of breeding and foraging habitats in farmland (Lefranc, 1997; Leugger-Eggimann, 1997). Moreover, some authors showed by modelling that potential food sources have a critical role in changes in local population size of the red-backed shrikes (Leugger-Eggimann, 1997; Schifferli et al., 1999; Kuper et al., 2000) and consequently used this knowledge in conservation programs for this declining and endangered species.

The red-backed shrike is mainly an insectivorous bird; sometimes the diet is supplemented by vertebrates (Lefranc & Worfolk, 1997). However, data on prey preferences is still lacking, except from one study in Spain (Hernandez et al., 1993). Moreover, data on the contribution of vertebrates to the diet of red-backed shrike are speculative, as studies focused on vertebrate use only, not within the context of the entire diet spectrum (Knysh, 1982; Hernandez, 1995).

Therefore, in this study we focused on (1) describing diet composition of the red-backed shrike in a farmland area, paying particular attention to the use of vertebrate prey and (2) prey selection.

MATERIAL AND METHODS

Study area and birds

The research was carried out during the period from July 1999 to August 2000 in the central Wielkopolska Province, western Poland, which is situated near town of Poznañ. The region is dominated by agricultural lands, and habitats include cultivated fields (80%), small forests (15%), marshlands (3%) and inhabited areas. Most red-backed shrikes arrive in the Wielkopolska region in early May (Tryjanowski & Sparks, 2001), start breeding between mid May and mid June (Kuźniak, 1999; Tryjanowski et al., 2000), and leave the study area in September or October. The study area was ca 60 km^2, with a mean density of 1.1 – 1.4 breeding pairs/km^2. For further details on the study area and the biology of the red-backed shrike see Kuźniak & Tryjanowski (2000) and Tryjanowski et al. (2000).

Food analyses

Three methods were applied simultaneously to analyse diet composition and to predict prey choice (Tryjanowski et al., 2003).

Food of nestlings was assessed by collar samples when the young were 5 to 10 days old. After every two to three feeding visits by the parents, the food was removed from the nestlings' throats with forceps. A total of 66 prey items were collected from 12 nests. Arthropods were preserved in ethanol (70%) and identified to the family level, using a reference collection obtained from the same study area (J. Karg, unpublished data).

Pellets were collected from 17 shrike territories. A total of 3832 prey items were identified (at least to family level) in 336 pellets. Pellets were analysed by a standard procedure (ROSENBERG & COOPER, 1990).

We also monitored larders and butchering points, where the shrikes prepare food for nestlings, as well as for themselves (LEFRANC & WORFOLK, 1997). We used data from 14 such points, with a total of 594 prey items removed from larders.

Available food

Because the red-backed shrikes prey mainly on invertebrates (for details see Results), only data for invertebrate prey were collected. A modified quick-trap method was used (RYSZKOWSKI & KARG, 1977). Samples were taken by covering 0.25 m^2 of the ground with a cage without a bottom (so called biocenometer). In six territories of red-backed shrike with places of higher shrike foraging activity, samples (10 in each control) were taken five times over the period June – August. Insects and spiders were collected from under the net by a vacuum sampler.

Data are presented as means for the whole study area during the breeding period, and are expressed as the number of individuals and/or biomass per m^2.

Calculations and statistics

Dry mass of invertebrates (after RYSZKOWSKI & KARG, 1977) was used for this analysis.

Prey choice analyses were carried out using compositional analysis of the five numerically dominant taxa : beetles (Coleoptera), bees and wasps (Hymenoptera), orthopterans (Orthoptera), bugs (Heteroptera), and a category containing all the remaining taxa referred to as "others" (moths and butterflies Lepidoptera, flies Diptera, spiders Araneae and many others – for details see Results).

To assess shrike selection of particular groups (orders) of insects, Ivlev's selectivity index (D) was calculated, as modified by JACOBS (1974) : D = (r-p)/(r+p-2rp), where r denotes the fraction of a given group of insects in the total number of insects identified from pellets, and p – the fraction of a group of insects in the insect community. D varies from –1 (negative selection) to 0 (catching proportional to abundance), to 1 (positive selection).

The analysis of prey choice was done in six territories only, where data on the red-backed shrike diet were collected using all three methods and the information on density of prey was available.

Because data on vertebrates in the red-backed shrike diet were limited, we decided to enlarge the study plot to include habitats located around it. Success of vertebrate catching was investigated using χ^2 - test, with two daily groups : with and without rain. Daily precipitation data for the study period were obtained from the Turew local meteorological station, located in the centre of the study area.

Standard statistical methods were used according to ZAR (1999). All statistical tests were two-tailed.

RESULTS

Invertebrates

Invertebrates constituted 98.9% of all (N = 4392) prey items, but 73.5% of dry mass from 415 g biomass consumed (Table in appendix). In general, the two methods of assessment of food contents, by number of individual prey items and by biomass, were highly positively correlated (r_s = 0.752, n = 150 prey categories, $p < 0.0001$).

Over 51% of the prey items were beetles; dung (Scarabeidae) and ground (Carabidae) beetles accounted for almost a third of the prey. Hymenoptera, with ants (Formicidae), ichneumon flies (Ichneumonidae), bees (Apidae) and wasps (Vespidae), made up around 22% of the diet. A third and a fourth group were grasshoppers and crickets (Orthoptera : Tettigonidae and Acridiidae) and bugs (Heteroptera), representing respectively 12.9 and 7.2% of the diet. The group labelled "others" invertebrates and vertebrates accounted together for only approximately 7%. Pattern of prey use by biomass is similar, although with more important roles for orthopterans and vertebrates (Fig. 1 A & B).

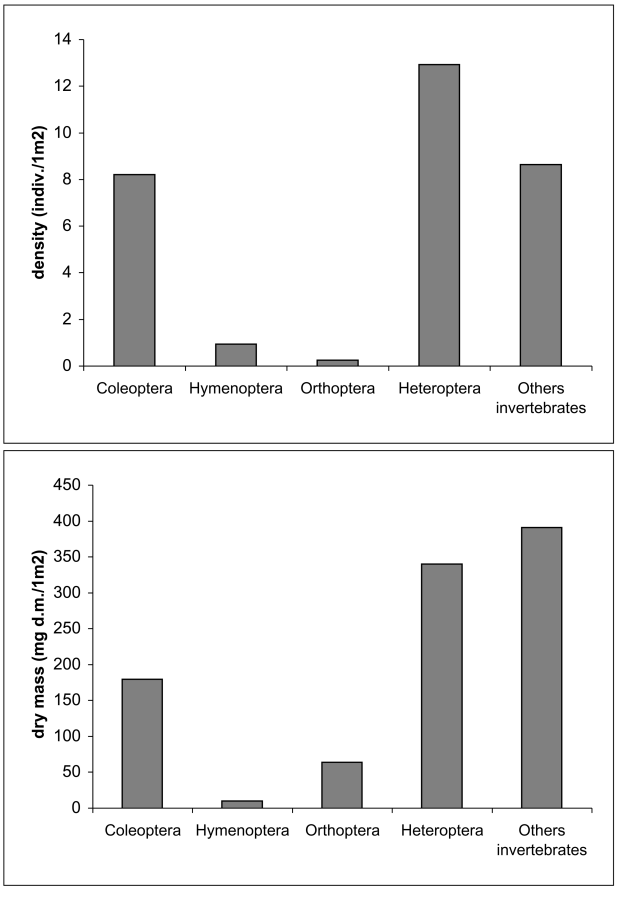

Fig. 1. – Percent composition of the red-backed shrike diet in terms of number of prey (A-top) and biomass (B-bottom).

Vertebrates

A total of 64 vertebrate prey caught by the red-backed shrike were recorded (Table 1). Small mammals constituted the main prey item (over 80%). Vertebrates were more often predated on cold and rainy days, than on days without rain ($p < 0.0001$).

TABLE 1

Vertebrates in the diet of the red-backed shrike in Wielkopolska Province, Poland. All methods complied.

Prey item	N individuals	%
Microtus sp.	29	45.3
Apodemus sp.	11	17.2
Mus musculus	1	1.6
Sorex sp.	11	17.2
Micromammalia total	*52*	*81.3*
Emberiza citrinella	4	6.3
Parus caeruleus	1	1.6
Passeriformes indet.	1	1.6
Aves total	*6*	*9.4*
Lacerta sp.	3	4.7
Rana sp.	3	4.7
Vertebrates total	64	100

Prey choice

The main potential food consisted of bugs (Heteroptera), by number of individuals, and other invertebrates (this category included mainly flies and dragonflies) by biomass (Fig. 2 A & B).

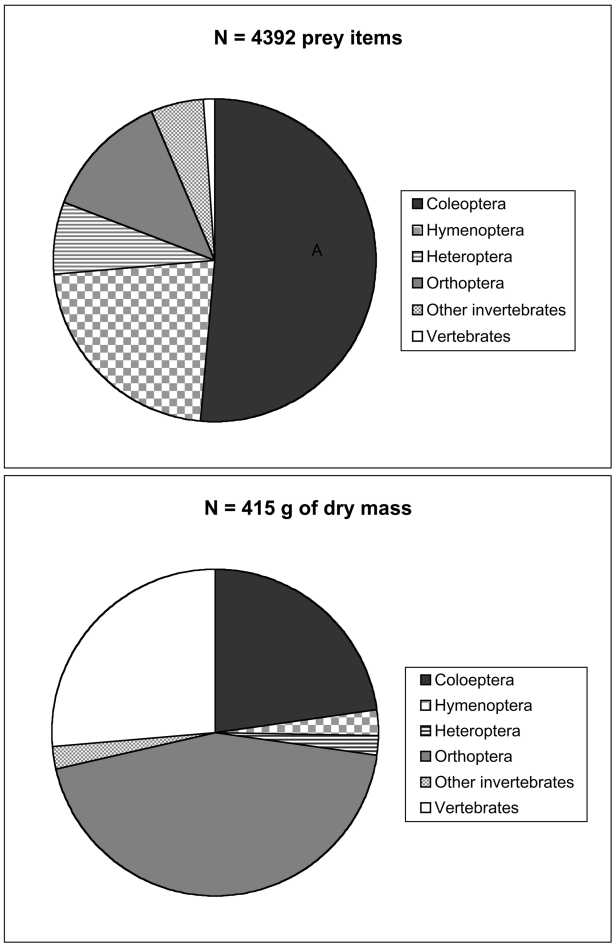

Fig. 2. – Density (top) and biomass (bottom) of potential invertebrate prey in territories of the red-backed shrike in Wielkopolska Province.

The red-backed shrike actual prey spectrum is significantly different from the potential one, both in terms of density of potential food ($p < 0.001$) and its biomass ($p < 0.001$). Out of five invertebrate taxa, Coleoptera, Hymenoptera and Orthoptera occurred in the content of red-backed shrike food more often than expected from sampling ("preferred food"), whereas Heteroptera and the category "other invertebrates" occurred less often ("avoided food"), both by numbers and by biomass (Table 2).

TABLE 2

The proportion of invertebrate groups in shrike territories and the proportion of prey selected by the red-backed shrikes in Wielkopolska Province, Poland. Explanations : PD – potential prey by density, PB – potential prey by biomass, SN – proportion among prey items in the red-backed shrike diet by number of specimens, SB – proportion of biomass given prey consumed. Index DD and Index DB - Ivlev's selectivity index for numbering and biomass, respectively.

Prey items	PD	SN	Index DD	Index PB	SB	Index DB
Coleoptera	0.265	0.615	0.632	0.182	0.420	0.530
Hymenoptera	0.030	0.218	0.799	0.010	0.073	0.775
Orthoptera	0.008	0.044	0.700	0.065	0.369	0.786
Heteroptera	0.418	0.077	-0.792	0.346	0.072	-0.744
Other invertebrates	0.279	0.046	-0.778	0.398	0.066	-0.807

DISCUSSION

Diet composition

The composition of the red-backed shrike diet is influenced by many external factors including habitat and territory quality, geographical location, weather conditions, time of day or season, as well as even by methods of food content analyses (Cramp & Perrins, 1993; Hernandez et al., 1993; Leugger-Eggimann, 1997; Tryjanowski et al., 2003).

The food composition of the red-backed shrike in Wielkopolska appears to be typical for its entire European range (review in : Cramp & Perrins, 1993). Especially large species of Coleoptera (Carabidae, Scarabeidae, Silphidae), Hymenoptera (*Apis*, *Vespa*, *Vespula*) and Orthoptera (*Tettigonia*) constituted a major part of the diet. Large species of beetles and orthopterans were found as the main red-backed shrike food in some areas (Randik, 1970; Cramp & Perrin,s 1993; Nikolov, 2002). These prey can also be potential shrike population density regulators (Kuper et al., 2000). Moreover, it is possible to improve the quality of breeding territories by establishing special habitat patches for these insects (Van Nieuwenhuyse et al., 1999; Kuper et al., 2000). Both beetles and grasshoppers, are captured by relatively energetically non-expensive methods, known as the sit-and-wait strategy (Lefranc & Worfolk, 1997, Van Nieuwenhuyse et al., 1999). Our findings showed the red-backed shrike is ecologically flexible in using new food sources. For example, in the study area the cereal ground beetle *Zabrus tenebrioides* forms an important part of the red-backed shrike diet. Imago of the cereal ground beetles live on the ears of the cereals. On many occasions the red-backed shrikes were observed hovering over ears of the cereal crops, especially during plagues of these beetles in 2000, when density was up to 2 indiv. / $1m^2$ (and biomass up to 175 mg / $1m^2$; J. Karg,

unpublished data). The shadow of birds causes beetles to roll off the plants as an antipredation reaction. They were then easily taken from the ground by the red-backed shrike.

As expected, the percentage of Hymenoptera in the diet (both by number and biomass) is high. However, as catching of bees and wasps is energetically costly (Leugger-Eggimann, 1997), these groups are probably less important for shrikes.

It is well known that vertebrates supplement the diet (Lefranc & Worfolk, 1997) and are represented by different taxa, ie. small passerines, small mammals, lizards, frogs and even fish (Knysh, 1982; Hernandez, 1995). So far only Mansfeld (1958) tried to explain why red-backed shrike seldom caught vertebrates. He suggested that it depends on vertebrate availability. For example, during rich vole years they constituted up to 12% of prey items (and up to 87% by biomass). In many other studies, however, both during rich- and poor-vole years, vertebrates constituted only small percentages of the prey (e.g. Lefranc & Worfolk, 1997). We support the theory that red-backed shrike catch vertebrates so infrequently because attacking and handling time for this prey are energetically expensive (Hernandez et al., 1993) while preying upon invertebrate appears to be a much less expensive hunting strategy. During cold and rainy weather, when insect activity is much reduced, red-backed shrikes are forced to prey on vertebrates.

Prey preferences

Habitat selection in birds follows a sequential hierarchy (Cody 1985). While studying food preference within territories, we should remember that a previous step was territory site choosing. However, in this process the red-backed shrike can use information related to potential food sources and their availability (e.g. height of plant cover, number of perches, vegetation structure), and availability of safe nesting sites (Kuźniak & Tryjanowski, 2000). The structure of potential food in the red-backed shrike territories is similar to the insect community in the whole farmland (Karg & Ryszkowski, 1996). It is linked with insect richness in margin habitats (small woodlots, hedgerows, ecotones, small open patches with xerophilic plants) and meadows, both preferred by breeding red-backed shrikes (Kuźniak & Tryjanowski, 2000).

Coleoptera, Hymenoptera and Orthoptera comprised preferred food of the red-backed shrike in the study area. In general, beetles were preferred, although these insects comprise a large and diverse group. It is well known that red-backed shrike prefer mostly large species (Cramp & Perrins, 1993; Lefranc & Worfolk, 1997), although they prey also upon small ones. Furthermore, among beetles we found species that secrete an odour, known as antipredator strategy (genus : *Silpha*, *Necrophorus*). Similarly, Mielewczyk (1976) found some individuals of the Colorado beetle *Leptinotarsa decemlineata* in the red-backed shrike diet. Other preferred groups, which use special antipredator strategies, were bees and wasps (Hymenoptera). The red-backed shrike efficiently caught these insects. Moreover, contrary to the suggestion by Gwinner (1961), bees sometimes are eaten, with their stings (often recorded in pellets).

In agreement with predictions (cf. Van Nieuwenheuse et al., 1999; Kuper at al., 2000) we found that Orthoptera species were also an important part of the diet. On the other hand, in Spain, Hernandez et al. (1993) indicated that grasshoppers and crickets are rather avoided, most probably due to local conditions.

Recommendations for management

Detailed knowledge of the food consumed may assist management programs for the red-backed shrike (Schfferli et al., 1999; Kuper et al., 2000). We conclude that the red-backed shrike is very flexible in its use of different food spectra. Therefore, not only the food source in an agricultural landscape is a potential regulatory factor for the red-backed shrike population, but also prey accessibility. To improve red-backed shrike foraging success, places with short vegetation and additional perches should to be prepared (Van Nieuwenhuyse et al., 1999). However, only management actions that focus on nest safety as well as foraging success can assist the red-backed shrike populations in modern farmland regions (Lefranc, 1997; Leugger-Eggimann, 1997; Schifferli et al., 1999).

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APPENDIX

Diet composition of the red-backed shrike during the breeding season in Wielkopolska Province, Poland. N denotes number of prey items (in number of individuals), whereas Prey is ratio of the total prey number, Bio is ratio of biomass (in mg of dry mass) consumed.

Prey item	N	Prey	Biomass	Bio
Coleoptera	2261	0.515	95072	0.229
Carabidae	1091	0.248	72323	0.174
Carabus violaceus	4	0.001	535	0.001
Carabus cancelatus	1	0.000	126	0.000
Carabus sp.	69	0.016	9225	0.022
Carabidae indet.	5	0.001	118	0.000
Broscus cephalotes	1	0.000	54	0.000
Bembidion sp.	70	0.016	82	0.000
Agonum sp.	5	0.001	42	0.000
Lebia sp.	1	0.000	26	0.000
Calosoma sp.	49	0.011	12332	0.030
Amara sp.	89	0.020	756	0.002
Calathus sp.	95	0.022	1617	0.004
Harpalus sp.	2	0.000	52	0.000
Idiochroma sp.	1	0.000	5	0.000
Ophonus sp.	14	0.003	119	0.000
Pterostichus sp.	397	0.090	21512	0.052
Pterostichus cupreus	1	0.000	54	0.000
Zabrus tenebrioides	287	0.065	25666	0.062
Scarabaeidae	719	0.164	7485	0.018
Anomala dubia	571	0.130	3023	0.007
Anomala aenea	1	0.000	42	0.000
Aphodius fimetarius	16	0.004	143	0.000
Aphodius rufus	1	0.000	9	0.000
Aphodius fossor	1	0.000	9	0.000
Aphodius sp.	15	0.003	101	0.000
Rhizotroous sp.	7	0.002	556	0.001
Onthophagus sp.	5	0.001	48	0.000
Valgus hemipterus	1	0.000	21	0.000
Geotrupes stercorosus	1	0.000	92	0.000
Geotrupes sp.	23	0.005	2105	0.005
Phyllopertha horticola	77	0.018	1336	0.003
Curculionidae	159	0.036	1083	0.003
Phyllobius sp.	82	0.019	300	0.001
Cleonus sp.	1	0.000	1	0.000
Otiorrhynchus sp.	16	0.004	596	0.001
Sitona sp.	17	0.004	79	0.000
Curculionidae indet.	43	0.010	107	0.000

Prey item	N	Prey	Biomass	Bio
Elateridae	59	0.013	810	0.002
Selatosomus aeneus	5	0.001	176	0.000
Agriotes sp.	27	0.006	261	0.001
Elateridae indet.	27	0.006	373	0.001
Staphylinidae	84	0.019	483	0.001
Lathrobium sp.	1	0.000	2	0.000
Ontholestes sp.	14	0.003	226	0.001
Philonthus sp.	46	0.010	66	0.000
Staphylinus sp.	5	0.001	164	0.000
Heterothops sp.	4	0.001	1	0.000
Staphylinidae indet.	14	0.003	25	0.000
Silphidae	63	0.014	10752	0.026
Nicrophorus sp.	32	0.007	8507	0.020
Silpha sinuata	1	0.000	26	0.000
Silpha sp.	18	0.004	468	0.001
Silphidae indet.	12	0.003	1751	0.004
Chrysomelidae	17	0.004	176	0.000
Lema sp.	3	0.001	9	0.000
Leptinotarsa decemlineata	1	0.000	74	0.000
Chrysomelidae indet.	13	0.003	93	0.000
Histeridae	12	0.003	84	0.000
Hister sp.	12	0.003	84	0.000
Cerambycidae	10	0.002	479	0.001
Spondylis buprestoides	2	0.000	17	0.000
Saperda sp.	1	0.000	58	0.000
Cerambycidae indet.	7	0.002	404	0.001
Leiodidae	3	0.001	0	0.000
Anisotoma humeralis	3	0.001	0	0.000
Cicindellidae	2	0.000	17	0.000
Cicindela sp.	1	0.000	8	0.000
Cicindela campestris	1	0.000	8	0.000
Lagriidae	3	0.001	112	0.000
Lagria hirta	3	0.001	112	0.000
Nitidulidae	4	0.001	2	0.000
Meligethes sp.	4	0.001	1	0.000
Others Coleoptera	37	0.008	1266	0.003

Prey item	N	Prey	Biomass	Bio
Hymenoptera	971	0.221	9872	0.024
Formicidae	291	0.066	258	0.001
Formica rufa	56	0.013	66	0.000
Lasius niger	145	0.033	91	0.000
Lasius sp.	7	0.002	4	0.000
Formica sp.	83	0.019	95	0.000
Ichneumonidae	328	0.075	992	0.002
Ophion sp.	31	0.007	247	0.001
Ophion luteus	3	0.001	24	0.000
Cryptinae	24	0.005	59	0.000
Ichneumonidae indet.	270	0.061	662	0.002
Apidae	157	0.036	3933	0.009
Apis mellifera	66	0.015	1416	0.003
Apis sp.	5	0.001	107	0.000
Andrena sp.	13	0.003	114	0.000
Bombus sp.	28	0.006	1420	0.003
Nomada sp.	1	0.000	4	0.000
Apidae indet.	44	0.010	872	0.002
Vespidae	153	0.035	3937	0.009
Vespula rufa	20	0.005	515	0.001
Vespula vulgaris	2	0.000	51	0.000
Vespula germanica	41	0.009	1055	0.003
Vespula sp.	75	0.017	1930	0.005
Vespa crabro	5	0.001	129	0.000
Vespa sp.	8	0.002	643	0.002
Dolichovespula norvegica	1	0.000	26	0.000
Dolichovespula sylvestris	6	0.001	154	0.000
Vespidae indet.	2	0.000	51	0.000
Myrmicidae	19	0.004	22	0.000
Myrmica sp.	19	0.004	22	0.000
Eumenidae	9	0.002	44	0.000
Sphecidae	3	0.001	50	0.000
Ammophila sabulosa	2	0.000	43	0.000
Sphecidae indet.	1	0.000	7	0.000
Mutillidae	1	0.000	2	0.000
Aryidae	1	0.000	2	0.000
Tenthredinidae	1	0.000	10	0.000
Chrysididae	1	0.000	3	0.000
Heteroptera	317	0.072	7823	0.019
Pentatomidae	286	0.065	7661	0.018
Palomena prasina	30	0.007	697	0.002
Palomena sp.	3	0.001	70	0.000
Pentatoma sp.	3	0.001	98	0.000
Picromerus bidens	2	0.000	65	0.000
Eurygaster maura	53	0.012	1922	0.005
Eurygaster sp.	6	0.001	218	0.001
Eurydema oleracea	2	0.000	16	0.000
Dolycoris sp.	7	0.002	81	0.000
Aelia acuminata	14	0.003	200	0.000
Aelia sp.	4	0.001	57	0.000
Pentatomidae indet.	162	0.037	4237	0.010
Lygaeidae	3	0.001	4	0.000
Acanthosomidae	2	0.000	16	0.000
Miridae	5	0.001	11	0.000
Notostira sp.	2	0.000	5	0.000
Lygus sp.	3	0.001	6	0.000
Coreidae	3	0.001	96	0.000
Coreus sp.	2	0.000	74	0.000
Coreidae indet.	1	0.000	22	0.000
Nabidae	16	0.004	32	0.000
Nabis ferus	3	0.001	6	0.000
Nabis sp.	13	0.003	26	0.000
Tingidae	1	0.000	0	0.000
Tingis sp.	1	0.000	0	0.000
Berytidae	1	0.000	1	0.000
Berytinus sp.	1	0.000	1	0.000
Orthoptera	568	0.129	183206	0.441
Tettigonidae	455	0.104	178211	0.429
Tettigonia sp.	434	0.099	177940	0.429
Metrioptera roselli	1	0.000	119	0.000
Tettigonidae indet.	10	0.002	152	0.000
Acridiidae	122	0.028	4955	0.012
Chorthippus sp.	30	0.007	1218	0.003
Acridiidae indet.	92	0.021	3736	0.009
Orthoptera indet.	1	0.000	41	0.000
Dermaptera	25	0.006	293	0.001
Forficulidae	25	0.006	293	0.001
Forficula auricularia	12	0.003	141	0.000
Chelidurella acanthopygia	1	0.000	12	0.000
Forficula sp.	2	0.000	23	0.000
Forficulidae indet.	10	0.002	117	0.000
Blattodea	1	0.000	2	0.000
Blattidae	1	0.000	2	0.000
Ectobius sp.	1	0.000	2	0.000
Odonata	10	0.002	5535	0.013
Diptera	87	0.020	805	0.002
Sarcophagidae	19	0.004	269	0.001
Calliphoridae	21	0.005	297	0.001
Lucilia sp.	1	0.000	14	0.000
Calliphoridae indet.	20	0.005	283	0.001
Tachinidae	12	0.003	286	0.001
Tachina sp.	1	0.000	3	0.000
Tachinidae indet.	8	0.002	27	0.000
Muscidae	2	0.000	12	0.000
Bibionidae	10	0.002	13	0.000
Asillidae	4	0.001	56	0.000
Diptera indet.	22	0.005	128	0.000
Lepidoptera	39	0.009	335	0.001
Noctuidae	3	0.001	24	0.000
Agrotis sp.	3	0.001	24	0.000
Lepidoptera larvae indet.	34	0.008	305	0.001
Lepidoptera indet.	2	0.000	5	0.000
Neuroptera	2	0.000	6	0.000
Myrmeleontidae	1	0.000	3	0.000
Myrmeleon sp.	1	0.000	3	0.000
Chrysopidae	1	0.000	3	0.000
Homoptera	4	0.001	14	0.000
Cercopidae	3	0.001	13	0.000
Philaenus sp.	3	0.001	13	0.000
Iassidae	1	0.000	1	0.000
Aphrodes sp.	1	0.000	1	0.000
Diplopoda	27	0.006	1804	0.004
Isopoda	9	0.002	192	0.000
Araneae	5	0.001	14	0.000
Opiliones	2	0.000	5	0.000
Molusca	12	0.003	6	0.000
Invertebrates total	**4343**	**0.989**	**304998**	**0.735**
Aves	3	0.001	18000	0.043
Mammalia	46	0.010	12000	0.029
Vertebrates total	**49**	**0.011**	**110000**	**0.265**

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Redescription critique des genres *Thryptodus*, *Pseudothryptodus* et *Paranogmius*, poissons marins (Teleostei, Tselfatiiformes) du Crétacé supérieur des Etats-Unis, d'Egypte et de Libye

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RÉSUMÉ. L'ostéologie de trois téléostéens marins du Crétacé, *Thryptodus* et *Pseudothryptodus* des Etats-Unis et *Paranogmius* d'Egypte et de Libye, est étudiée. Ces trois genres appartiennent à l'ordre des Tselfatiiformes. *Thryptodus* et *Pseudothryptodus* sont des genres valides très différents de *Bananogmius*, un autre tselfatiiforme avec lequel ils ont souvent été mis en synonymie. Au sein des Tselfatiiformes, *Thryptodus* est plus particulièrement proche de *Plethodus* et à un degré moindre de *Martinichthys*. *Pseudothryptodus* est également apparenté à *Thryptodus* mais en diffère par l'architecture moins spécialisée de son museau. *Paranogmius* est un autre genre valable, caractérisé notamment par la présence sur le neurocrâne d'une fosse subtemporale, une structure primitive déjà disparue chez les autres Tselfatiiformes, et d'un vomer très élargi antérieurement.

MOTS CLEFS : *Thryptodus*, *Pseudothryptodus*, *Paranogmius*, Tselfatiiformes, Teleostei, Crétacé supérieur marin, Etats-Unis, Egypte, Libye, ostéologie.

On the genera *Thryptodus*, *Pseudothryptodus* and *Paranogmius*, marine fishes (Teleostei, Tselfatiiformes) from the Upper Cretaceous of the United States, Egypt and Libya

ABSTRACT. The osteology of three marine Upper Cretaceous teleosts, *Thryptodus* and *Pseudothryptodus*, from the U.S.A., and *Paranogmius*, from Egypt and libya, is studied. These genera belong to the order Tselfatiiformes. *Thryptodus* and *Pseudothryptodus* are valid genera quite different from *Bananogmius*, another tselfatiiform fish with which they are often synonymized. Within the Tselfatiiformes, *Thryptodus* appears more specially related to *Plethodus* and less closely to *Martinichthys*. *Pseudothryptodus* is also related to *Thryptodus* but differs from this genus by its snout architecture less specialized. *Paranogmius* is another valid genus, characterized more particularly by the presence of a subtemporal fossa on the neurocranium, a primitive structure already lost by all other Tselfatiiformes, and of an anteriorly enlarged vomer.

KEY WORDS : *Thryptodus*, *Pseudothryptodus*, *Paranogmius*, Tselfatiiformes, Teleostei, marine Upper Cretaceous, U. S. A., Egypt, Libya, osteology.

INTRODUCTION

Les Tselfatiiformes sont, avec les Ichthyodectiformes et les Pachyrhizodontoidei, l'un des trois grands groupes de téléostéens qui ont dominé les mers durant le Crétacé et qui n'ont plus de descendants dans les ichthyofaunes marines actuelles. Ce sont des poissons de taille moyenne à grande, dont la forme du corps évoque celle des Scombridae et des Coryphaenidae. Le crâne est presque toujours médio-pariétal. Les dents des mâchoires et du palais sont minuscules et groupées en plages. Les os dentés sont perforés de fins canalicules très caractéristiques de l'ordre. Les nageoires pectorales sont insérées haut sur les flancs. Les nageoires pelviennes, en position abdominale, sont souvent réduites. Les nageoires dorsale et anale sont longues et hautes. La nageoire caudale compte 19 rayons principaux et l'hypurostégie est prononcée. Les rayons des nageoires sont branchus mais rarement segmentés. Le complexe urophore comporte une plaque hypurale résultant de la fusion des quatre premiers hypuraux, plaque

elle-même soudée à un petit centre terminal ural 1-2. Pour davantage d'informations anatomiques concernant les Tselfatiiformes, je renvoie à TAVERNE, 2000a.

L'ordre compte près de vingt genres. Certains les regroupent en une unique famille des Plethodidae (PATTERSON, 1993 : 627), tandis que d'autres les répartissent en deux familles, les Plethodidae et les Tselfatiidae (NELSON, 1994 : 90). La révision de tous ces genres n'étant pas encore achevée, il n'est guère possible aujourd'hui de trancher la question.

Les Tselfatiiformes ont vécu dans la Mésogée eurafricaine, le Paléoatlantique et la mer intérieure nord-américaine. On trouve leurs restes fossiles dans les gisements de l'Aptien au Campanien en Europe, dans le nord de l'Afrique, en Amérique du Nord et dans le nord de l'Amérique du Sud.

TAVERNE (2000a : 14-20) a montré que les Tselfatiiformes étaient des Clupeocephala archaïques, plus primitifs qu'un clade qui réunit les Clupeomorpha, les Ostariophysi et les Euteleostei, soit tous les autres Clupeocephala ayant encore des représentants dans la nature actuelle. Cette position systématique des Tselfatiiformes a été confirmée par l'analyse cladistique de CAVIN (2001 : fig. 16).

TAVERNE (1975, 1983, 1999, 2000a, b, c, d, 2001a, b, c, 2002a, b, sous presse) a entrepris la révision des Tselfatiiformes ainsi que l'étude de leurs relations phylogénétiques et intergénériques. Le présent article s'inscrit dans cette série de travaux. Il porte sur trois genres de cet ordre : *Thryptodus* et *Pseudothryptodus*, créés par LOOMIS (1900 : 229, 235) pour des téléostéens du Crétacé supérieur marin du Kansas (Etats-Unis), et *Paranogmius*, innové par WEILER (1935 : 32) pour un poisson du Cénomanien inférieur de l'Egypte. Deux de ces genres, *Thryptodus* et *Pseudothryptodus*, ont souvent été tenus par la suite pour de simples synonymes de *Bananogmius* (qui a remplacé *Anogmius* préoccupé, cf. TAVERNE, 2001b), un autre représentant des Tselfatiiformes (STEWART, 1900 : 391; HAY, 1903 : 29; APPLEGATE, 1970 : 414, 416).

La description originale de *Thryptodus* se basait sur un crâne à peu près complet pour l'espèce-type *Thryptodus zitteli*, crâne long d'environ 20 cm (LOOMIS, 1900 : fig. 6, pl. XXI). En comparant les longueurs respectives de la tête et du corps chez les quelques Tselfatiiformes connus en entier (ARAMBOURG, 1943, 1954; BARDACK & TELLER-MARSHALL, 1980; TAVERNE, 1983, 1999, 2001a, 2001b), on peut estimer que le poisson complet devait approcher le mètre de longueur totale. En ce qui concerne la seconde espèce, *Thryptodus rotundus*, ainsi que pour *Pseudothryptodus intermedius*, l'unique espèce de ce genre, seuls quelques os sont connus (LOOMIS, 1900 : pl. XXII, fig. 1-8). Malheureusement, le matériel type de ces deux genres *Thryptodus* et *Pseudothryptodus*, conservé au Musée de Munich, a été détruit durant la Seconde Guerre Mondiale (P. WELLNHOFER, in litt. 15/02/1974). Les planches de LOOMIS (1900) sont cependant très précises et elles permettent de comprendre les données ostéologiques relatives à ces poissons. On peut ainsi aujourd'hui rectifier les diverses erreurs d'interprétation de LOOMIS (1900) grâce aux connaissances qui ont été acquises sur le crâne des Tselfatiiformes ces dernières années. De plus, certains fragments de museau de *Thryptodus zitteli* ont été découverts depuis dans le Campanien inférieur de l'Alabama, dans le Cénomanien du Texas ainsi que dans le Cénomanien et le Coniacien du Kansas (APPLEGATE, 1970 : 416; TAVERNE, 2000c : fig. 1E, F, G; SHIMADA & SCHUMACHER, sous presse) qui autorisent aujourd'hui une meilleure compréhension de cette structure que ne l'avait LOOMIS (1900).

Rappelons encore que JORDAN (1905 : 44) avait créé une famille des Thryptodontidae pour les deux genres *Plethodus* et *Thryptodus*, famille qu'il considérait comme proche parente des Albulidae. Ce taxon familial n'a plus été utilisé par la suite jusqu'à ce que PATTERSON (1993 : 627) le place en synonymie des Plethodidae.

Quant à *Paranogmius* et son unique espèce, *Paranogmius doederleini*, le matériel de la description originale comportait deux neurocrânes incomplets, quelques os crâniens, des vertèbres et une partie de squelette caudal (WEILER, 1935 : fig. 4-6, pl. III, fig. 8, 16, 26). Tous ces restes étaient de très grande taille. L'un des deux neurocrânes, quoique dépourvu du mésethmoïde, mesurait 45 cm de long. La comparaison avec les quelques Tselfatiiformes entiers connus, indique un poisson qui dépassait 3 mètres de longueur totale. Cela en fait le plus grand de tous les Tselfatiiformes et l'un des plus grands téléostéens de tous les temps. D'autres restes d'un tselfatiiforme géant ont été découverts dans les terrains cénomaniens de la région et appartiennent certainement au même genre, aucun autre représentant de l'ordre n'atteignant cette taille. Il s'agit d'un fragment de mâchoire supérieure à laquelle est fixé un morceau de dermopalatin et d'une partie d'un os denté provenant de Libye (QUAAS, 1902 : pl. XXVIII, fig. 16, 17) ainsi que d'un morceau de cleithrum, d'un bout de dentaire et d'un vomer trouvés en Egypte (STROMER, 1936 : fig. 3, pl. I, fig. 9, 10). Le matériel relatif à *Paranogmius* a également été détruit durant la Seconde Guerre Mondiale (SCHAAL, 1984 : 4). Seules les descriptions et les figures de ces auteurs nous permettent encore d'interpréter ce poisson géant. Néanmoins, les connaissances acquises actuellement sur le squelette des Tselfatiiformes autorisent dans ce cas-ci aussi à tirer d'intéressantes conclusions qui ne pouvaient guère apparaître aux auteurs anciens.

Le but du présent travail est donc de revoir l'ostéologie de *Thryptodus zitteli*, *Pseudothryptodus intermedius* et *Paranogmius doederleini* et de préciser leurs rapports avec les autres genres et espèces de l'ordre.

Quant à *Thryptodus rotundus*, TAVERNE (2000c : 116) a montré qu'il n'appartenait pas au genre *Thryptodus* mais devait s'intégrer au genre *Plethodus*. Je ne traiterai donc pas de cette espèce dans les pages qui suivent.

MATÉRIEL ET MÉTHODES

Le matériel étudié ci-après provient de l'American Museum of Natural History de New York (AMNH) et du Musée de Paléontologie des Vertébrés de l'Université du Kansas à Lawrence (KUVP). Toutes ces pièces se rapportent à l'espèce *Thryptodus zitteli* et proviennent des terrains de la Niobrara Formation (Coniacien à Campanien) du Kansas (Etats-Unis).

AMNH 19557 : un museau isolé (désigné ici comme néotype, l'holotype étant détruit; figuré dans TAVERNE, 2000c : fig. 1E, F, G).

KUVP 456, 457 et 459 : trois museaux isolés.

KUVP 25899 : la moitié gauche d'un très gros museau isolé.

Le matériel a été examiné à l'aide d'une loupe et d'un stéréomicroscope WILD M5 muni d'une chambre claire. Les dessins ont été réalisés par l'auteur.

ABRÉVIATIONS SUR LES FIGURES

AN :	angulaire (= angulo-splénial)
ANT :	antorbitaire
ART :	articulaire
BO :	basioccipital
BRSTG :	rayons branchiostèges
CHY a. :	cératohyal antérieur
CHY p. :	cératohyal postérieur (= épihyal)
DBHY :	dermobasihyal (= dermentoglosse)
DBBR 1-3 :	dermobasibranchial unique des trois premiers arcs branchiaux
DBBR 4 :	dermobasibranchial du quatrième arc branchial
DETH :	dermethmoïde (= rostral)
DN :	dentaire (= dento-splénial, dentalo-splénial)
DPAL :	dermopalatin
DSPH :	dermosphénotique
ECPT :	ectoptérygoïde
ENPT :	entoptérygoïde (= endoptérygoïde, mésoptérygoïde)
EP :	épiotique (= épioccipital)
EXO :	exoccipital
FR :	frontal
HHY d. :	hypohyal dorsal
HHY v. :	hypohyal ventral
IC :	intercalaire
IOP :	interoperculaire
IORB 2 :	deuxième infraorbitaire
LETH :	ethmoïde latéral
METH :	mésethmoïde
MX :	maxillaire
NA :	nasal
OP :	operculaire
OSPH :	orbitosphénoïde
PA :	pariétal
PMX :	prémaxillaire
POP :	préoperculaire
PRO :	prootique
PS :	parasphénoïde
PSPH :	pleurosphénoïde (= ptérosphénoïde)
PTE :	ptérotique
QU :	carré (= quadratique)
RART :	rétroarticulaire
SETH :	supraethmoïde
SMX :	supramaxillaire
SOC :	supraoccipital
SORB :	supraorbitaire
SPH :	sphénotique (= autosphénotique)
V1 :	première vertèbre
VO :	vomer (= prévomer)
d. f. :	*dilatator fossa*
f.a. :	fenêtre auditive
f. hyom. :	fossette articulaire neurocrânienne pour l'hyomandibulaire
f. st. :	fosse subtemporale
f. t. :	fosse temporale (= fosse posttemporale)
f. V :	ouverture antérieure de la *pars jugularis* par laquelle passent le nerf trijumeau (V) et la veine jugulaire
f. t. h. VII :	ouverture postérieure de la *pars jugularis* par laquelle passent le *truncus hyoideomandibularis* du nerf facial (VII) et la veine jugulaire
f. X :	foramen du nerf vague (X)
p. j. :	*pars jugularis*

OSTÉOLOGIE DE *THRYPTODUS ZITTELI* LOOMIS, 1900 (Fig. 1-3)

Les os crâniens dermiques sont très ornementés, comme c'est d'ailleurs le cas chez la plupart des Tselfatiiformes.

Le museau est extrêmement massif. Il est formé des prémaxillaires, du mésethmoïde et du vomer. Le mésethmoïde est un os allongé, large, surtout dans sa région postérieure, et plat. Son bord antérieur forme un épais bourrelet ventral de forme irrégulière. Cette zone épaisse représente la partie endochondrale de l'os, c'est-à-dire le supraethmoïde, tandis la large pellicule osseuse dorsale en est la partie dermique, c'est-à-dire le dermethmoïde ou rostral (TAVERNE, 2000c : fig. 1G). Le mésethmoïde est complètement recouvert par les prémaxillaires qui deviennent énormes et dont les processus dorsaux rejoignent les frontaux avec lesquels ils s'articulent. Selon les exemplaires, les deux prémaxillaires sont fusionnés en une pièce unique (AMNH 19557 et LOOMIS, 1900 : pl. XXI, fig. 1) ou simplement ankylosés de façon ferme l'un à l'autre avec leur suture qui demeure visible (KUVP 456, 457, 459). Le bord ventral des prémaxillaires ou du prémaxillaire unique porte une large plage denticulée et se recourbe sous le vomer dont il recouvre le bord antérieur. Les ethmoïdes latéraux sont très développés et en contact avec le mésethmoïde. Le vomer est court, extrêmement large et édenté. Les nasaux sont énormes, parfaitement intégrés au reste du toit crânien et articulés avec les prémaxillaires et les frontaux.

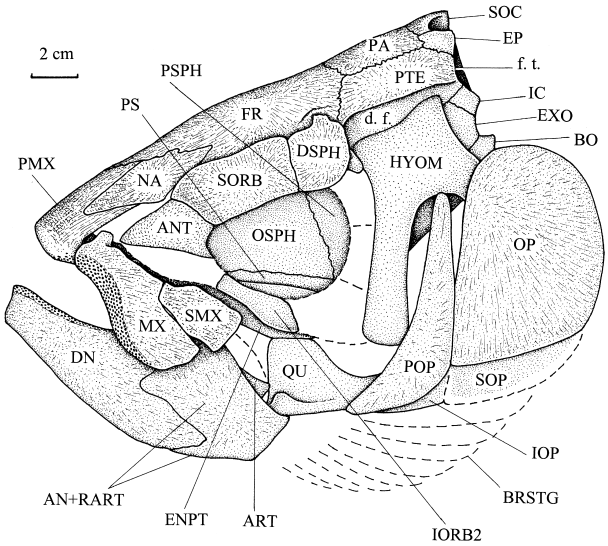

Fig. 1. – *Thryptodus zitteli* LOOMIS, 1900. Crâne en vue latérale gauche (modifié d'après LOOMIS, 1900 : fig. 6 et pl. XXI, fig. 1, 2, 5, 7).

Le toit crânien est large, quasi aussi large à l'avant qu'à l'arrière, relativement plat et dépourvu de dépression médiane clairement marquée. Les frontaux sont vastes, ainsi que les pariétaux plus ou moins quadrangulaires et en contact médian, formant ainsi un crâne de type médiopariétal. Les ptérotiques sont allongés et bordent latéralement les pariétaux. Le supraoccipital est court, large et porte une petite crête médiane. Les épiotiques touchent le supraoccipital et forment une sorte de protubérance à l'arrière du crâne. Les sphénotiques possèdent un proces-

sus postorbitaire en forme d'ergot qui dépasse latéralement le bord du frontal.

La *dilatator fossa* est allongée, peu élevée, située sur la face ventrale du crâne et portée par le sphénotique et le ptérotique. La fossette articulaire neurocrânienne pour l'hyomandibulaire est longue et nettement plus large à l'avant qu'à l'arrière. Elle est creusée dans le sphénotique, le prootique et le ptérotique. La fosse temporale (= posttemporale) est située à l'arrière du neurocrâne et s'ouvre entre le ptérotique, l'épiotique et l'intercalaire. Il n'y a pas de fosse subtemporale.

L'orbitosphénoïde très développé et les pleurosphénoïdes, nettement plus petits, s'articulent avec le parasphénoïde, formant ainsi un septum interoculaire osseux complet. Le parasphénoïde est très large et sa vaste plaque denticulée est pyriforme et légèrement concave. La largeur maximale de cette plaque représente les 65 % de sa longueur. L'éventuel basisphénoïde n'est pas visible.

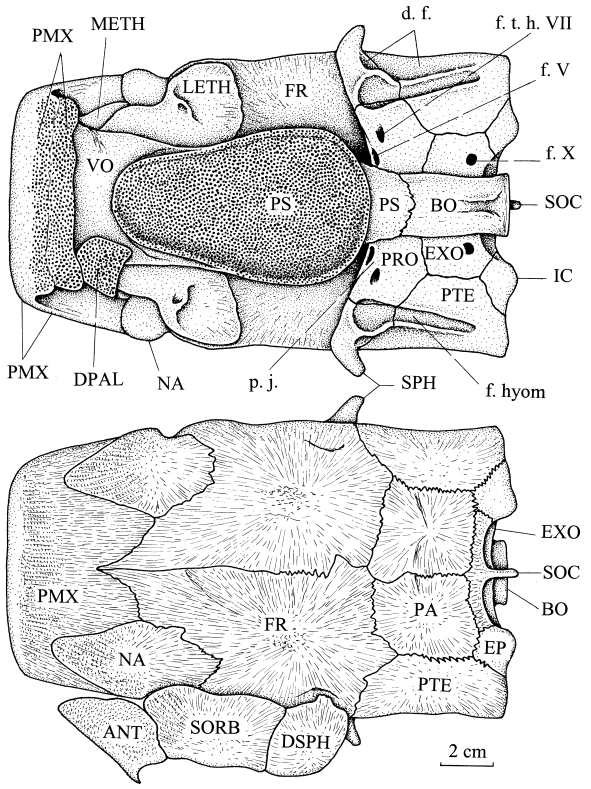

Fig. 2. – *Thryptodus zitteli* Loomis, 1900. Neurocrâne en vue ventrale (au-dessus) et en vue dorsale (en dessous) (modifié d'après Loomis, 1900 : pl. XXI, fig. 1-3). Le basioccipital est ajouté d'après mes observations sur d'autres crânes de Tselfatiiformes.

Les prootiques, la portion ventrale des ptérotiques, les exoccipitaux, les intercalaires forment l'arrière du plancher neurocrânien. Le basioccipital n'est pas conservé. La *pars jugularis*, percée dans le prootique et par où transite la veine jugulaire, est courte; son ouverture antérieure sert au passage du nerf trijumeau (V) et la postérieure à l'émergence du *truncus hyoideomandibularis* du nerf facial (VII). Le foramen du nerf vague (X) perce l'exoccipital.

Du cercle des os circumorbitaires, seuls sont connus le deuxième infraorbitaire, étroit et allongé, ainsi que l'antorbitaire, le supraorbitaire et le dermosphénotique, trois os de grande taille, articulés entre eux et avec le bord latéral du nasal et du frontal. Le coin ventro-postérieur de l'antorbitaire forme une petite pointe. Le dermosphénotique couvre le sphénotique mais ne déborde pas sur le ptérotique.

Outre le prémaxillaire que nous avons déjà évoqué, chaque hémi-mâchoire supérieure comprend le maxillaire et un seul supramaxillaire. Le maxillaire est assez court et s'articule sur le prémaxillaire. Son bord buccal porte une large plage denticulée qui déborde à la fois sur les faces internes et externes de l'os. Le supramaxillaire se loge dans une encoche du bord dorsal du maxillaire. Chaque hémi-mandibule comporte le dentaire, l'articulaire autogène, l'angulaire et le rétroarticulaire soudés l'un à l'autre ainsi que le coronoméckelien. Le bord oral du dentaire porte une plage denticulée qui déborde fortement sur la face interne de l'os, surtout vers l'arrière (Loomis, 1900 : pl. XXI, fig. 5). L'articulation mandibulo-quadratique se situe au niveau du milieu de l'orbite.

Le palatin est court, large et denticulé. L'entoptérygoïde est long, large et sa face interne est complètement denticulée. Loomis (1900 : pl. XXI, fig. 8) a erronément identifié l'entoptérygoïde comme le cinquième cératobranchial. L'ectoptérygoïde n'est pas connu. Le carré est triangulaire, renflé au niveau de son condyle articulaire pour la mandibule et son processus quadratique est tout entier situé postérieurement au corps-même de l'os.

Les os de la série operculaire comprennent le préoperculaire, l'interoperculaire et l'operculaire. Le sous-operculaire est incomplet. Ces os sont relativement petits par rapport à la taille du crâne. Le préoperculaire montre une branche dorsale longue et une branche ventrale très courte

L'arc hyoïdien est connu presque complètement. L'hyomandibulaire est large dorsalement et s'étire ventralement en une forte tige osseuse. Son *processus opercularis* est court mais très épais. Le symplectique est une petite baguette osseuse. La barre hyoïdienne se compose d'un hypohyal dorsal et d'un hypohyal ventral, tous deux très développés, d'un cératohyal antérieur large et dépourvu de foramen béryciforme ainsi que d'un très court cératohyal postérieur. L'interhyal n'est pas connu. La plaque denticulée linguale est vaguement ovale et légèrement convexe. Sa largeur maximale vaut les 65 % de sa longueur. Cette plaque se compose de deux pièces articulées entre elles et de développement à peu près égal. La première pièce est le dermobasihyal (= dermentoglosse) et le seconde représente les dermobasibranchiaux des trois premiers arcs branchiaux soudés entre eux. Il n'y a pas de dermobasibranchial du quatrième arc, à l'encontre du cas des Tselfatiiformes primitifs (Taverne, 2000b : fig. 10, 2001b : fig. 13). Le premier pharyngobranchial est petit et édenté (Loomis, 1900 : pl. XXI, fig. 3 où l'on distingue ce petit os articulé à l'arrière du parasphénoïde). L'urohyal est étroit et allongé. En coupe, il montre une forme en « T » renversé. Le reste du squelette branchial est inconnu.

On ne connaît rien non plus du squelette postcrânien si ce ne sont les huit premières vertèbres. Elles sont creusées de quatre petits puits, deux dorsaux et deux ventraux dans lesquels s'articulent par gomphose respectivement les arcs neuraux et hémaux.

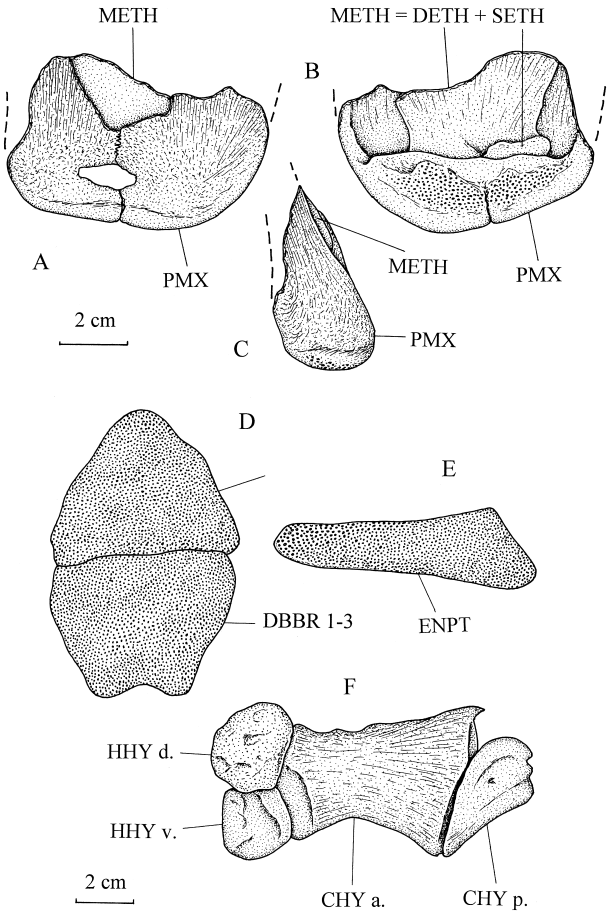

Fig. 3. – *Thryptodus zitteli* Loomis, 1900. Museau de l'exemplaire KUVP 459 en vue dorsale (A), ventrale (B) et latérale droite (C); cet exemplaire illustre le cas où les deux prémaxillaires sont fermement ankylosés mais pas encore fusionnés. Plaque denticulée linguale (D). Entoptérygoïde droit en vue interne (E). Barre hyoïdienne gauche en vue externe (F). (D, E, F modifiés d'après Loomis, 1900 : pl. XXI, fig. 4, 6, 8).

OSTÉOLOGIE DE *PSEUDOTHRYPTODUS INTERMEDIUS* LOOMIS, 1900 (Fig. 4)

Les prémaxillaires sont libres l'un par rapport à l'autre, de forme trapézoïde, aussi hauts que larges, avec un processus ascendant très développé et de même largeur que le corps de l'os proprement dit. La surface externe de l'os est très ornementée. Les très hauts processus ascendants des deux prémaxillaires devaient forcément recouvrir une grande partie du mésethmoïde sous-jacent mais qui, lui, n'est pas connu. Le bord oral de l'os se replie en une courte aile ventrale qui porte une large plage denticulée et qui devait chevaucher le bord antérieur du vomer, lequel n'est pas non plus connu, à la manière des prémaxillaires de *Thryptodus*.

Les os que Loomis (1900 : pl. XXII, fig. 5, 6, 7) interprète comme les troisième, quatrième et cinquième cératobranchiaux ne ressemblent guère aux cératobranchiaux des Tselfatiiformes. Ceux-ci sont nettement moins larges, en forme de gouttière et jamais denticulés (obs. pers.). Il semble clair que ces trois os erronément interprétés par Loomis (1900) sont respectivement un grand fragment du

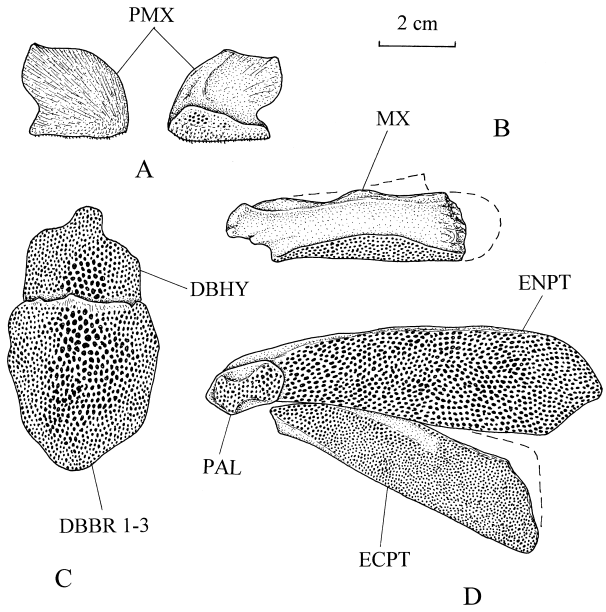

Fig. 4. – *Pseudothryptodus intermedius* Loomis, 1900. Prémaxillaire droit en vue externe et interne (A). Maxillaire gauche en vue externe (B). Plaque denticulée linguale (C). Palatin, entoptérygoïde et ectoptérygoïde droits en vue interne (D). (A, B, C et D modifiés d'après Loomis, 1900 : pl. XXII, fig. 3-7).

maxillaire, l'ectoptérygoïde et l'entoptérygoïde accompagné du dermopalatin.

Le maxillaire est relativement court. Son extrémité antérieure se renfle en un condyle articulaire pour le prémaxillaire et pour le palatin. Le bord oral de l'os porte une large plage denticulée.

L'entoptérygoïde et l'ectoptérygoïde sont des os larges et allongés, le premier l'étant encore plus que le second. Le dermopalatin est petit et articulé à l'extrémité antérieure de l'entoptérygoïde. Les trois os sont denticulés sur toute leur surface.

La plaque denticulée linguale est large, vaguement ovale, moins large toutefois que chez *Thryptodus zitteli* puisque sa largeur maximale ne représente que les 56 % de sa longueur. Cette plaque comporte deux pièces articulées l'une à l'autre et d'inégale grandeur : un dermobasihyal plutôt court suivi d'un dermobasibranchial des trois premiers arcs branchiaux, nettement plus allongé. Le dermobasibranchial du quatrième arc, primitivement présent chez les Tselfatiiformes (Taverne, 2000b : fig. 10, 2001b : fig. 13), fait ici défaut comme chez *Thryptodus*.

Quelques fragments d'os circumorbitaires, le carré et quelques vertèbres ont été découverts. Loomis (1900 : 235) précise que ces pièces ressemblent à leurs homologues chez *Thryptodus* mais il ne les illustre pas.

Rien d'autre n'est connu du squelette de ce poisson.

OSTEOLOGIE DE *PARANOGMIUS DOEDERLEINI* WEILER, 1935 (Fig. 5)

Les os dermiques sont ornés de petits trous et de fines crêtes qui rayonnent parfois à partir d'un centre. Cependant cette ornementation n'est pas très prononcée sur le toit crânien, à l'encontre d'autres Tselfatiiformes.

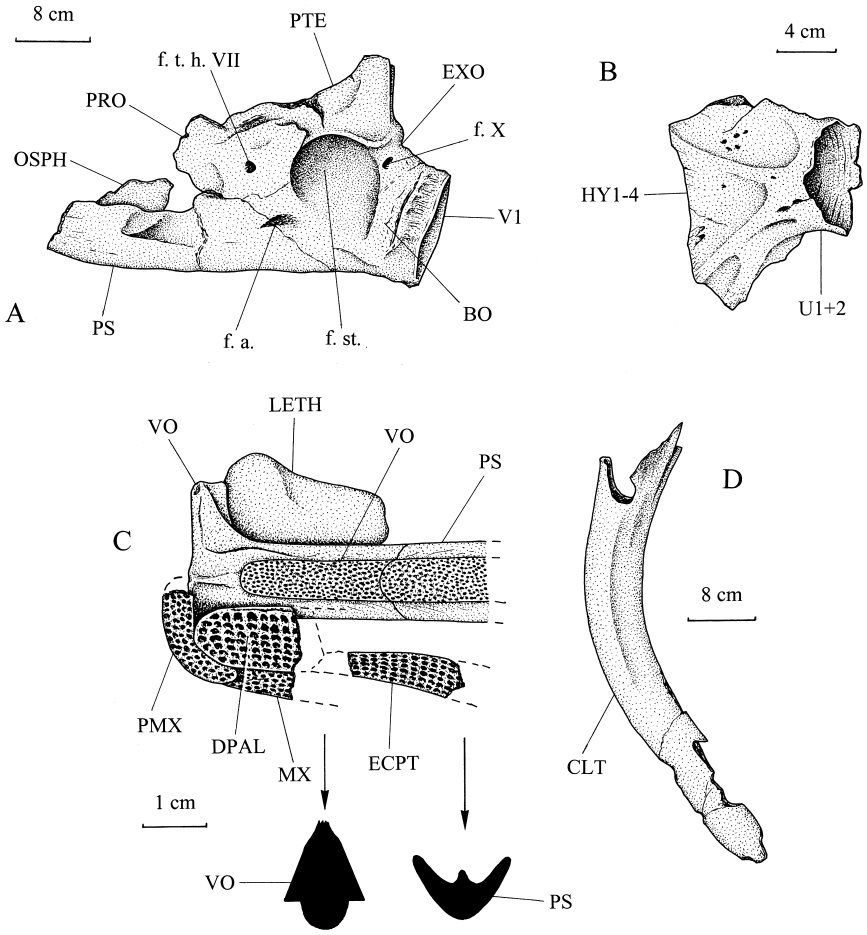

Fig. 5. – *Paranogmius doederleini* Weiler, 1935. (A) partie postérieure du neurocrâne en vue latérale gauche (modifié d'après Weiler, 1935 : fig. 4). (B) partie du squelette caudal (modifié d'après Weiler, 1935 : fig. 6). (C) reconstitution du palais, avec (en bas) une coupe schématique du vomer et du parasphénoïde (modifié d'après Quaas, 1902 : pl. XXVIII, fig. 16a, 17, Weiler, 1935 : fig. 4a et Stromer, 1936 : pl. I, fig. 9a, b). (D) partie du cleithrum (modifié d'après Stromer, 1936 : fig. 3).

Le mésethmoïde n'est pas connu mais devait être large compte tenu de la position quasi à angle droit du prémaxillaire par rapport au maxillaire (Quaas, 1902 : pl. XXVIII, fig.16) et de l'exceptionnelle largeur de l'extrémité antérieure du vomer. Un os épais et massif situé à côté du vomer et figuré par Weiler (1935 : pl. III, fig. 8, 16) ne peut être qu'un très gros ethmoïde latéral. Le vomer est abîmé et tordu sur le seul neurocrâne qui possède encore cet os mais on peut néanmoins observer que sa partie antérieure est extrêmement large (ibid. : pl. III, fig. 8, 16). Le vomer isolé montré par Stromer (1936 : pl. I, fig. 9a, b) est mieux conservé. Il a la forme d'un « T », tant son extrémité antérieure est élargie par rapport au reste de l'os. Il porte en son milieu une longue plage denticulée, guère large mais à surface très convexe, ce qui est exceptionnel chez les Tselfatiiformes.

Le toit crânien est relativement plat, formé essentiellement par les vastes frontaux et les pariétaux. Ceux-ci sont grands, plus ou moins quadrangulaires et jointifs, déterminant ainsi un crâne de type médio-pariétal. Le supraoccipital paraît petit, pointu à l'arrière et dépourvu de crête médiane (Weiler, 1935 : pl. III, fig. 8) mais il s'agit peut-être d'un artefact de fossilisation, l'os n'étant que partiellement conservé. Il n'y a pas de dépression fronto-pariétale. Seuls une petite partie des ptérotiques est préservée. Les sphénotiques et les épiotiques sont inconnus.

La surface denticulée du parasphénoïde n'est pas très large mais elle montre un relief très convexe (ibid. : fig. 4a), unique chez les Tselfatiiformes. Cela correspond donc bien à la forme de la zone denticulée du vomer et permet la parfaite articulation des plages denticulées de ces deux os. Le parasphénoïde est dépourvu de processus basiptérygoïde mais porte une petite crête médio-dorsale que rejoint l'orbitosphénoïde, délimitant de la sorte un septum osseux interorbitaire complet, comme chez les autres Tselfatiiformes. Les pleurosphénoïdes et l'éventuel basisphénoïde ne sont pas connus.

Le foramen du *truncus hyoideomandibularis* du nerf facial (VII) s'ouvre au milieu de la face latérale du prootique. Il ne semble pas y avoir d'ouverture postérieure particulière sur le prootique pour la veine jugulaire qui passait donc probablement aussi par ce foramen facial. Il y a une petite fenêtre auditive à la limite du parasphénoïde, du prootique et du basioccipital. Une vaste fosse subtemporale se creuse dans la face ventrale du neurocrâne, dans la région du prootique et de l'exoccipital, juste sous le ptérotique. La conservation de cette structure téléostéenne

primitive est exceptionnelle chez les Tselfatiiformes. L'exoccipital, percé par le foramen du nerf vague (X), ne participe pas à la formation du condyle articulaire pour le squelette axial, condyle formé tout entier par le grand basioccipital. La première vertèbre est soudée au basioccipital mais la ligne de suture demeure encore visible.

Le supraorbitaire et le dermosphénotique (= deuxième supraorbitaire de WEILER, 1935 : 33) sont articulés entre eux et avec le frontal. Les autres os orbitaires ne sont pas connus.

Une grande partie de l'arc palatin est perdue. On connaît cependant le dermopalatin, accolé au prémaxillaire et au maxillaire (QUAAS, 1902 : pl. XXVIII, fig. 16a). C'est un os plat, assez large et couvert de petits puits, restes de l'implantation d'une plage de petites dents. Un autre os de cet arc, en forme de plaque allongée, assez étroit et également porteur sur toute sa surface de traces d'implantation de petites dents est également connu (ibid. : pl. XXVIII, fig. 17). Il est nettement plus étroit que le dermopalatin. Il s'agit donc de l'ectoptérygoïde, puisque l'entoptérygoïde, lui, aurait été aussi large, voire plus large que le dermopalatin. Le carré est triangulaire et garni d'un gros condyle articulaire (WEILER, 1935 : fig. 5).

Les mâchoires ne sont que très partiellement conservées (QUAAS, 1902 : pl. XXVIII, fig. 16a, b; STROMER, 1936 : pl. I, fig. 10). Le prémaxillaire et le maxillaire portent chacun sur leur bord oral une large plage couverte de petits puits qui servaient à l'implantations des dents. Le bord oral du dentaire est garni d'une plage de denticules. Le bord symphysaire de l'os est élevé et porte une sorte de petit ergot à son extrémité dorsale, juste en avant de la zone denticulée.

Le préoperculaire ressemble à celui de *Bananogmius* (WEILER, 1935 : 37), large, avec des branches dorsale et ventrale bien développées et de longueur subégale.

Le cleithrum est grand, étroit sur toute sa longueur, avec une branche dorsale courte et une branche ventrale longue (STROMER, 1936 : fig. 3), ce qui est typique des Tselfatiiformes.

Les premières vertèbres sont hautes, étroites, avec des faces latérales ornées de fines stries horizontales et des hémapophyses autogènes réduites à de petits nodules osseux et articulées dans des petites fossettes de la face ventrale des corps vertébraux correspondants (WEILER, 1935 : fig. 5).

Le squelette caudal comporte une hémi-vertèbre terminale résultant de la fusion des centres uraux 1 et 2. Les quatre premiers hypuraux sont soudés en une large plaque hypurale, elle-même fusionnée à la petite vertèbre terminale (ibid. : fig. 6). On ne distingue pas de gouttière marquant sur cette plaque la séparation des hypuraux dorsaux et ventraux.

DISCUSSION

Thryptodus, *Pseudothryptodus* et *Paranogmius* au sein des téléostéens

La morphologie générale du crâne, les grands pariétaux quadrangulaires et jointifs, la dentition buccale et intrabuccale faite de plages de denticules, l'aspect ponctué des os à dents lorsque ces dernières sont perdues, l'antorbitaire, le supraorbitaire et le dermosphénotique articulés entre eux et avec le bord du frontal, le septum interoculaire osseux, l'articulation du supramaxillaire dans une encoche du bord supérieur du maxillaire, l'angulaire et le rétroarticulaire soudés, l'articulaire autogène sont autant de caractères qui, alliés, témoignent que *Thryptodus* appartient à l'ordre des Tselfatiiformes.

Les mêmes plages denticulées sur les mâchoires, les os ptérygoïdes et la plaque linguale, l'aspect ponctué de ces os à dents et la forme particulière des prémaxillaires permettent de ranger également *Pseudothryptodus* dans cet ordre.

La dentition des mâchoires et du palais formée de plages de denticules, l'aspect ponctué des os dentés lorsque les dents sont perdues, le crâne médio-pariétal, le septum interoculaire osseux, le supraorbitaire et le dermosphénotique articulés entre eux et au frontal, l'aspect du cleithrum et la partie connue du squelette caudal sont autant d'éléments qui attestent que *Paranogmius* est, lui aussi, un membre des Tselfatiiformes.

Thryptodus, *Pseudothryptodus* et *Bananogmius*

FIELITZ & SHIMADA (1999) ont décrit il y a peu l'ostéologie d'une nouvelle espèce de *Bananogmius* et TAVERNE (2001b) vient d'effectuer la révision de ce genre. Ces deux travaux autorisent aujourd'hui la comparaison détaillée avec *Thryptodus* et *Pseudothryptodus*. On peut ainsi vérifier si ces deux genres sont ou non synonymes du premier.

Chez *Bananogmius*, les prémaxillaires ne sont ni hypertrophiés, ni soudés ou ankylosés l'un à l'autre et ils ne recouvrent pas non plus la totalité du mésethmoïde et l'extrémité antérieure du vomer. Le vomer n'est pas élargi et il est denticulé. La plaque denticulée parasphénoïdienne est allongée, modérément élargie et de forme rectangulaire. La plaque linguale est large mais sans aucune tendance à s'arrondir et, de plus, composée de trois pièces puisque le dermobasibranchial du quatrième arc est conservé. La branche ventrale du préoperculaire est allongée. Les os de la série operculaire sont de grande taille par rapport au reste du crâne. L'antorbitaire porte un processus ventro-postérieur pointu très développé. Le supraorbitaire est modérément élargi. Ce sont là autant de traits qui différencient clairement *Bananogmius* de *Thryptodus*.

Quant à *Pseudothryptodus*, le seul fait de posséder un ectoptérygoïde bien développé et denticulé sur toute sa face interne suffit à le distinguer de *Bananogmius* dont l'une des apomorphies est justement d'avoir l'ectoptérygoïde réduit et édenté. Le prémaxillaire de *Bananogmius* ne montre pas le repli ventral qui existe chez *Pseudothryptodus*. La forme et la composition de la plaque linguale diffère aussi dans ces deux genres.

Thryptodus et *Pseudothryptodus* ne sont donc pas, à l'encontre de ce que beaucoup ont pensé (STEWART, 1900 : 391; HAY, 1903 : 29; APPLEGATE, 1970 : 414, 416), des synonymes de *Bananogmius*.

Ils méritent par contre chacun un statut générique particulier car certains de leurs caractères les distinguent de tous les autres genres connus dans l'ordre.

La diagnose amendée de *Thryptodus*

La diagnose amendée de *Thryptodus* devient : tselfatiiforme de taille moyenne; museau très large et très massif; prémaxillaires extrêmement développés, soudés ou ankylosés fermement l'un à l'autre, recouvrant complètement le mésethmoïde, articulés avec les frontaux et repliés ventralement en une aile osseuse qui porte la plage denticulée et qui chevauche l'extrémité antérieure du vomer; nasaux hypertrophiés et articulés avec les prémaxillaires et les frontaux; vomer très élargi et édenté; toit crânien très large, assez plat et dépourvu de dépression médiane fronto-pariétale clairement marquée; crâne médiopariétal avec de grands pariétaux quadrangulaires; supraoccipital court, large et orné d'une crête médiane; plaque denticulée du parasphénoïde très large et pyriforme; processus postorbitaire du sphénotique en forme d'ergot; *dilatator fossa* longue, étroite et creusée sur le sphénotique et le ptérotique; fossette articulaire pour l'hyomandibulaire allongée, large à l'avant, étroite à l'arrière et creusée sur le sphénotique, le prootique et le ptérotique; *pars jugularis* étroite; antorbitaire, supraorbitaire et dermosphénotique très larges, articulés les uns aux autres et avec le frontal; coin postéro-ventral de l'antorbitaire formant une petite pointe; septum interoculaire osseux complet; mâchoire supérieure courte; plage denticulée du maxillaire débordant sur les deux faces de l'os; supramaxillaire articulé dans une encoche du bord dorsal du maxillaire; palatin denticulé; entoptérygoïde denticulé; mandibule modérement allongée; dentaire à bord oral garni d'une plage denticulée; angulaire et rétroarticulaire fusionnés; articulaire autogène; articulation mandibulo-quadratique située au niveau du milieu de l'orbite; os de la série operculaire petits par rapport à la taille du crâne; plaque denticulée linguale ovale, constituée de deux pièces de surface à peu près égale, le dermobasihyal et le dermobasibranchial des trois premiers arcs; dermobasibranchial du quatrième arc absent; hypohyaux très développés; cératohyal postérieur très court; arcs neuraux et hémaux articulés par gomphose sur les centre vertébraux.

La diagnose amendée de *Pseudothryptodus*

La diagnose amendée de *Pseudothryptodus* devient : tselfatiiforme de taille moyenne; prémaxillaires séparés l'un de l'autre, à processus ascendant très large et très haut, recouvrant partiellement le massif mésethmoïdien, à bord ventral recourbé qui porte une plage denticulée et qui recouvre l'extrémité antérieure du vomer; maxillaire court portant une large plage denticulée; palatin, entoptérygoïde et ectoptérygoïde bien développés et complètement denticulés; plaque denticulée linguale plus ou moins ovale, composée de deux pièces, un petit dermobasihyal et un grand dermobasibranchial des trois premiers arcs.

Les affinités de *Thryptodus* au sein des Tselfatiiformes (Fig. 6, 7)

Les relations intergénériques au sein des Tselfatiiformes n'ont pas encore fait l'objet d'une étude détaillée puisque la révision des différents genres de l'ordre n'est pas encore achevée. On peut néanmoins déjà faire quelques constatations intéressantes.

Thryptodus appartient au groupe majoritaire des Tselfatiiformes à toit crânien relativement plat et non pas à celui des formes spécialisées à toit crânien courbe en section transversale. Son préoperculaire à branche ventrale raccourcie le situe en position apomorphe par rapport à des genres tels *Bananogmius*, *Luxilites* et *Niobrara* qui possèdent encore un préoperculaire aux branches dorsale et ventrale bien développées et de longueur subégale (Taverne, 2001a : fig. 3-4, 2001b : fig. 2, 2002 : fig. 1-3).

D'autre part, l'architecture si particulière du museau de *Thryptodus* se retrouve chez un autre tselfatiiforme, le genre *Plethodus* malgré de légères différences de forme. Chez ce dernier également, les prémaxillaires hypertrophiés et soudés l'un à l'autre recouvrent le mésethmoïde, tandis qu'un large rebord ventral de l'os porte la plage denticulée et devait chevaucher l'extrémité antérieure du vomer (Taverne, 2000c : fig. 1A, B, C, D). De plus, *Plethodus* montre, comme *Thryptodus*, des plaques denticulées parasphénoïdienne et linguale très développées et devenues ovales (Woodward, 1899 : pl. XIII, fig. 1-4). Il est donc clair qu'au sein des Tselfatiiformes *Thryptodus* et *Plethodus* sont des genres très proches parents. On ne peut cependant pas les confondre puisque *Thryptodus* possède encore une plaque denticulée linguale composée du dermobasihyal et du dermobasibranchial articulés mais distincts l'un de l'autre, tandis que ces deux pièces sont complètement fusionnées chez *Plethodus*.

Parmi les Tselfatiiformes, le genre *Martinichthys*, remarquable par son rostre, présente aussi des affinités particulières avec *Thryptodus* et *Plethodus*. Les prémaxillaires y sont également hypertrophiés, plus ou moins soudés l'un à l'autre, recouvrant non seulement le mésethmoïde mais aussi le vomer, formant ainsi une sorte de faux palais (Taverne, 2000b : fig. 1-6, 9). C'est donc là une spécialisation encore plus avancée des prémaxillaires. *Martinichthys* montre aussi un élargissement considérable de la plaque denticulée linguale mais il diffère cependant de *Thryptodus* et *Plethodus* par la conservation d'un dermobasibranchial du quatrième arc (Taverne, 2000b : fig. 10C, D) déjà perdu chez ces deux genres. *Martinichthys* ne montre cependant pas d'élargissement de la plaque denticulée parasphénoïdienne (ibid. : fig. 5, 6) à l'encontre de *Thryptodus* et *Plethodus*.

Cette parenté entre *Martinichthys* et *Thryptodus* semble confirmée par la découverte encore inédite dans le Crétacé supérieur du Kansas d'un nouveau tselfatiiforme à long museau qui paraît intermédiaire entre ces deux genres (K. Shimada, *in lit*. 27/9/2002, 2/10/2002, 11/12/2002 et 20/12/2002).

Les affinités de *Pseudothryptodus* au sein des Tselfatiiformes (Fig. 6)

Quoique très imparfaitement connu, *Pseudothryptodus* paraît proche parent de *Thryptodus* et de *Plethodus* bien que moins spécialisé que ces derniers. Ses deux prémaxillaires demeurent indépendants l'un de l'autre mais le processus d'hypertrophie qui va permettre à ces deux os de recouvrir le mésethmoïde et l'extrémité antérieure du vomer est déjà en place. A ce niveau, *Pseudothryptodus* se montre intermédiaire entre les Tselfatiiformes classiques, tels *Bananogmius*, où les prémaxillaires laissent

apparaître le mésethmoïde et le bout du vomer et les genres *Thryptodus*, *Plethodus* et *Martinichthys*. Le net élargissement et la forme vaguement ovale de sa plaque denticulée linguale est un autre trait anatomique qui apparente *Pseudothryptodus* à *Thryptodus* et *Plethodus*, et plus spécialement au premier puisque cette plaque demeure composée de deux pièces.

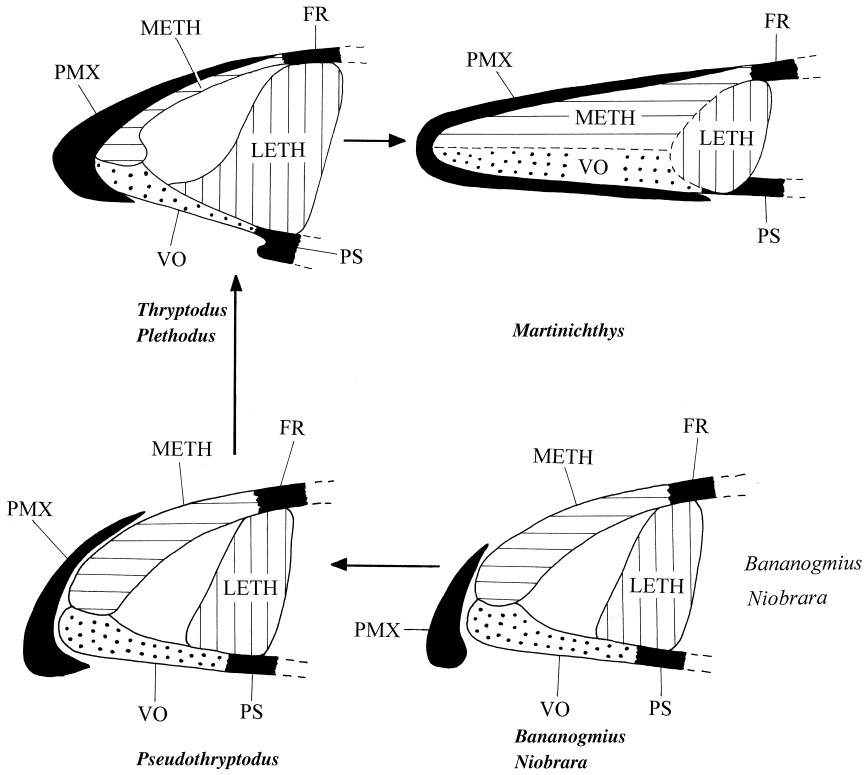

Fig. 6. – L'évolution du museau et de l'encapuchonnement progressif du mésethmoïde et du vomer chez les Tselfatiiformes, à partir de genres primitifs, tels *Bananogmius* et *Niobrara*, en passant par *Pseudothryptodus*, puis par *Thryptodus* et *Plethodus* pour aboutir enfin à *Martinichthys*.

La validité du genre *Paranogmius*

Outre sa taille gigantesque, *Paranogmius* se distingue des autres Tselfatiiformes par une plésiomorphie, la conservation d'une fosse subtemporale, et deux apomorphies, la forme en « T » du vomer et la surface convexe des régions denticulées du vomer et du parasphénoïde. Chez tous les Tselfatiiformes dont la face ventrale du neurocrâne est connue, la fosse subtemporale, cette structure téléostéenne archaïque, est perdue. Aucun autre tselfatiiforme ne possède non plus le curieux vomer en forme de « T » de *Paranogmius*. Quant aux plages denticulées du vomer et du parasphénoïde des autres Tselfatiiformes, elles sont toujours plates ou concaves, jamais convexes. *Paranogmius* est donc un genre valable.

La diagnose amendée de *Paranogmius*

La diagnose amendée de *Paranogmius* devient : tselfatiiforme géant atteignant 3 mètres de long; museau large; toit crânien assez plat; grands pariétaux jointifs; supraoccipital peu développé; dents des mâchoires disposées en plages; bord symphysaire du dentaire élevé et garni d'un petit ergot dorsal; dermopalatin vaste, plat et denticulé; ectoptérygoïde denticulé; grand vomer en forme de « T » suite à l'élargissement considérable de l'extrémité antérieure; zones denticulées du vomer et du parasphénoïde à surface convexe; fosse subtemporale présente; supraorbitaire et dermosphénotique articulés entre eux et avec le frontal; orbistosphénoïde rejoignant le parasphénoïde et déterminant un septum interoculaire osseux complet; préoperculaire à branches dorsale et ventrale bien développées et subégales; cleitrhum étroit, à branches dorsale courte et ventrale longue; centres uraux 1 et 2 fusionnés en une hémi-vertèbre terminale à laquelle se soude une plaque hypurale résultant de la fusion des quatre premiers hypuraux.

Les affinités de *Paranogmius* au sein des Tselfatiiformes

Paranogmius appartient lui aussi au groupe majoritaire des Tselfatiiformes à toit crânien plus ou moins plat. Son préoperculaire est du type le plus primitif au sein de l'ordre, avec des branches dorsale et ventrale bien développées et de longueur subégale, comme chez *Bananogmius*, *Luxilites* et *Niobrara*. Son ethmoïde latéral de grande taille est un autre trait primitif dans l'ordre. De plus, *Paranogmius* est le seul tselfatiiforme qui possède encore une fosse subtemporale, cette structure téléostéenne archaïque que l'on trouve chez les Elopomorpha et les Osteoglossomorpha. Même les genres généralement considérés comme les plus primitifs des

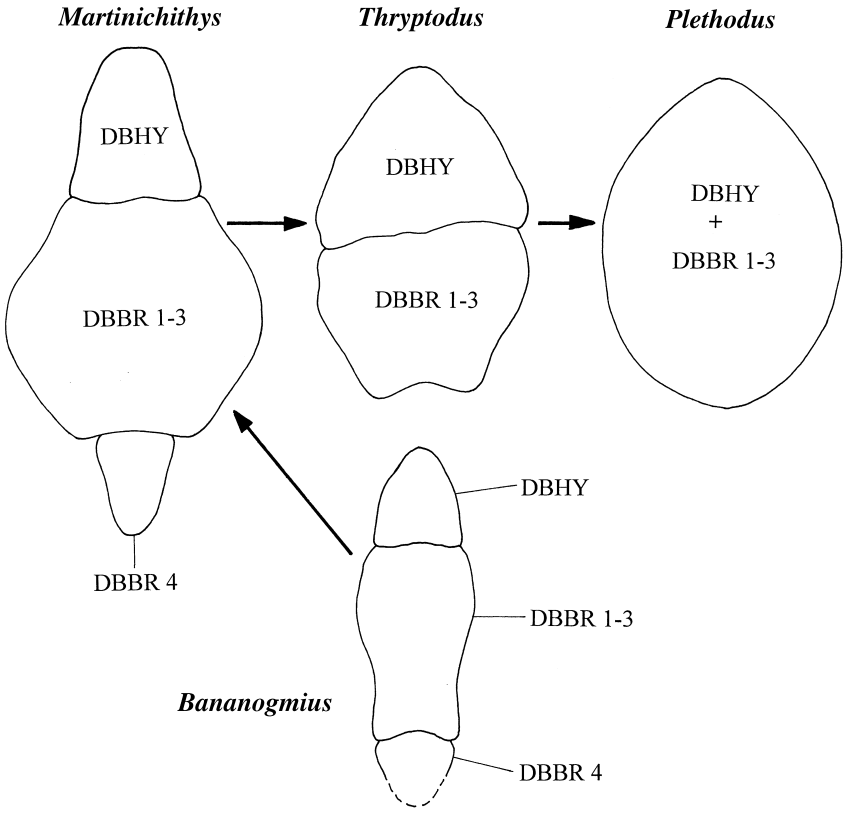

Fig. 7. – L'évolution de la plaque denticulée linguale chez les Tselfatiiformes, à partir de genre primitifs, tels *Bananogmius*, en passant par *Martinichthys*, puis par *Thryptodus* et enfin par *Plethodus*. On y note l'élargissement progressif de la plaque qui s'ovalise dans ces trois derniers genres, la perte du dermobasibranchial du quatrième arc chez *Thryptodus* et *Plethodus* ainsi que la fusion entre le dermobasihyal et le dermobasibranchial des trois premiers arcs chez *Plethodus*.

Tselfatiiformes, tels *Bananogmius* ou *Niobrara* (Taverne, 2001a : fig. 4, 2001b : fig. 10) ont déjà perdu la fosse subtemporale. On peut donc en conclure que *Paranogmius* est moins évolué encore que ces deux genres-là, ce qui en fait assurément l'un des représentants les plus primitifs de l'ordre. Par contre, la disparition chez *Paranogmius* de la gouttière qui marque sur la plaque hypurale la séparation entre les hypuraux dorsaux et ventraux paraît situer ce poisson en position apomorphe par rapport à *Eoplethodus* qui est le genre possédant le squelette caudal le plus primitif de tous les Tselfatiiformes (Taverne, 2000d : fig. 1).

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Biogeographical observations on four scolytids (Coleoptera, Scolytidae) and one lymexylonid (Coleoptera, Lymexylonidae) in Wallonia (Southern Belgium)

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ABSTRACT. Following a very sudden, early and deep frost at the end of autumn 1998, the availability of weakened trees (mainly beech trees) reached very high levels in Southern Belgium in the spring of 1999. Consequently, the ambrosia beetles *Trypodendron domesticum* L. and *T. signatum* (Fabricius) (Coleoptera, Scolytidae) initiated outbreaks and, in 2000 and 2001, they heavily contributed to the depreciation of nearly 1,600,000 m^3 of stem volume (to upper limit girth of 22 cm) in the natural regions of "Ardenne" and "Belgian Lorraine". Because of the lack of biogeographical data on both insects, of their conspicuous aggressiveness towards apparently healthy trees and of the economic importance of the beech wood chain in Belgium, a large-scale survey was undertaken in 2001, in order to outline the range of both ambrosia beetles in Wallonia. To this effect, a network of 172 traps baited with ethanol was set up, attempting to cover the Walloon beech forest as representatively as possible. Two other scolytids and one lymexylonid were also frequently caught, which made it possible to outline their regional distribution too. Although the damage was limited to the Ardenne and Belgian Lorraine, *T. domesticum* and *T. signatum* are widespread throughout Wallonia. We discuss these results, their long-term validity, the secondary pest status of these insects and the need for a permanent monitoring of the major forest pest species and diseases.

KEY WORDS : Scolytidae, *Trypodendron domesticum*, *T. signatum*, *Xyleborus dispar*, *Taphrorychus bicolor*, Lymexylonidae, *Hylecoetus dermestoides*, range, Wallonia, beech disease, monitoring.

INTRODUCTION

The beech forests of Southern Belgium have been undergoing severe entomological and fungal attacks in 2000 and 2001. Many factors, such as repeated droughts, destabilisation of the root systems as a consequence of the storms of 1990, air pollution and soil impoverishment, for instance, may potentially be involved in and have encouraged this situation. However, the factor that most likely set off the crisis seems to be a very early, sudden and severe frost that happened during the autumn of 1998, after a period of mild temperatures (HUART & RONDEUX, 2001). This climatic event induced cortical and subcortical lesions. Produced by anaerobic fermentation, the subsequent ethanol emissions triggered off the response of wood-boring insects, mainly scolytids.

Two ambrosia beetles[1] were responsible for the entomological damage : *Trypodendron domesticum* L. 1758 and *T. signatum* (Fabricius, 1787). Although apparently not as problematic as both *Trypodendron* species, the population levels of a third ambrosia beetle, *Xyleborus dispar* (Fabricius, 1792), were very high too. Their role in the past crisis still remains unclear (GRÉGOIRE & DE PROFT, 2002, unpubl. report).

Almost the totality of the beetle attacks occurred on beech trees, *Fagus sylvatica* L.. However, very sporadic attacks occurred on another thin-barked tree species : the sycamore, *Acer pseudoplatanus* L.. RONDEUX et al. (2002) showed that the occurrence of the damage was restricted to the natural regions of "Ardenne" and "Belgian Lorraine", almost exclusively at elevations higher than 350 m a.s.l. (Fig. 1). According to this study, more than 11 % of the beech trees (girth at breast height > 39 cm) situated in those regions were damaged (by scolytids and/or fungi); in some plots, however, the scolytid attack rates reached 100%. The damage amounted to nearly 1,600,000 m^3 stem volume [2] (18 % of the total standing volume) with huge financial losses as a result of the depreciation of the wood and premature tree felling. Although not easily quantifiable, the financial losses for private and public forest owners undoubtedly exceeded 50 million m^3.

While in 2000 the entomological attacks were restricted to very weakened trees, both *Trypodendron* spp. begun in the spring of 2001 to attack healthy-looking beech trees. This conspicuous aggressiveness bewildered the Walloon forest managers since :

1. the financial incomes associated with the beech wood chain are of major importance in Belgium;
2. *T. domesticum* and *T. signatum* are polyphagous and able to breed on several tree species producing highly

[1] Wood-boring Scolytoidae (Coleoptera) feeding upon symbiotic fungi.

[2] To upper limit girth of 22 cm.

valuable timber such as oaks, maples, ash, etc. (BALACHOWSKY, 1949; LEKANDER et al., 1977);

3. although the most important part of the Belgian beech forest is situated in the Ardennes, a significant part of it is also scattered throughout Wallonia : it was thus feared that the attack range could extend to the rest of this Region.

Very little biogeographical information on scolytids is available for Wallonia. The most valuable is undoubtedly the maps drawn by DOUROJEANNI in 1971. Unfortunately, besides being relatively ancient, those maps are based on few, randomly and non-synchronously collected observations (since he drew them up from museum specimens) : some regions of Wallonia may thus have been under-sampled and some data could be out-of-date.

In this context, a survey aimed at outlining the present range of *T. domesticum* and *T. signatum* in Wallonia was undertaken. According to LEKANDER et al. (1977), accurate data on the distribution of scolytids are always needed since "many of them are of direct concern to the forest manager". This was undoubtedly the case of *T. domesticum* and *T. signatum* in Wallonia. In particular, up-to-date biogeographical information on those scolytids were, to some extent, of interest for improving the understanding of the "Walloon beech crisis" and the subsequent set up of a pest control program.

X. dispar, *Taphrorychus bicolor* (Herbst, 1793) (Coleoptera, Scolytidae), and another prejudicial and very polyphagous wood borer, *Hylecoetus dermestoides* (L., 1761) (Coleoptera, Lymexylonidae), were also regularly caught, which made it possible to outline their distribution too.

MATERIAL AND METHODS

Window traps of transparent plastic (10 x 23 cm high) with funnel and collector vial were hooked on beech trees at 1.7 m height above ground level. The trees belonged to different categories of diameter. Ethanol, which is known to attract many bark and ambrosia beetles such as e.g. *Trypodendron* spp. (e.g. MOEK, 1970; KERK, 1972; BAUER & VITÉ, 1975; NIJHOLT & SCHÖNHERR, 1976; KLIMETZEK et al., 1986; SCHROEDER & LINDELÖW, 1989; BYERS, 1992; MARKALAS & KALAPANIDA, 1997), was released from a plastic-vial dispenser (ca. 250 mg.day^{-1}). These were made of polyethylene (2 cm diam. x 8 cm high). A total of 172 traps were set up from the beginning of April to the end of October 2001.

The chosen location of most of the traps was among the Walloon Permanent Forest Inventory (W.P.F.I.) plots. The W.P.F.I. is a network of 11,000 permanent plots located on a 500 x 1,000 m systematic grid covering Wallonia. It aims at assessing the wood resources of the Region and monitoring the sustainability of the forest management (see e.g. LECOMTE & RONDEUX, 1994). Globally, 222 plots were extracted from the W.P.F.I. data base : they concern "public beech stands (with beech relative basal area (G_{beech}) > 66 %) of at least 1 ha". One trap was set up in each of 129 of those plots ("W.P.F.I. traps"). These were selected in order to cover Wallonia as homogeneously as possible. However, the number of plots (and consequently of the traps) is much greater in the Ardennes and Belgian Lorraine, where the forestation rate is much higher than in the other natural regions of Wallonia (LECOMTE & RONDEUX, 1995). Thus, in order to get a better picture of the beetles' range, 43 additional traps were set up in the less-forested natural regions ("non-W.P.F.I. traps"). Fig. 2 shows the trap network and the W.P.F.I. plots located in beech stands (relative G_{beech} > 50 %).

Catches were checked every 8th week. Because of the small size of the traps and the number of traps per site (one), only the presence of a species at a given site was recorded (i.e. at least one individual caught), instead of the number of individuals. The survey methodology doesn't permit assessment of the local population levels of the insects (see HENIN et al., 2003). For the same reasons, the absence of a species in the collections at a site may not be considered as evidence of the absence of this species at this site. Therefore, the equality of observed presence rates (number of sites where a given species was caught, vs. number of trapping sites in a particular natural region or altitude category) was not tested statistically (either within a species or between species).

The insects were identified using BALACHOWSKY's identification key (1949) and the reference collection of the entomological museum of the Zoology Department (Faculté universitaire des Sciences agronomiques, Gembloux).

RESULTS

Fig. 3 and Fig. 4 show our observations of *T. domesticum* and *T. signatum* in Wallonia. Both species occur all over this Region, whatever the altitude or the natural region. *T. domesticum* was caught in 144 traps : it was thus present in at least 84 % of the sites. *T. signatum* was caught in 90 traps (presence rate of min. 52 %).

As shown on Fig. 5, *X. dispar* is also widely distributed in Wallonia. It was caught in 119 traps, which means it was present in at least 69 % of the sites. As for both *Trypodendron* spp., altitude and natural region do not influence the presence of the insect.

Based on our survey, the distribution of *T. bicolor* appears relatively wide (Fig. 6). This bark beetle was caught in 65 traps (presence rate of min. 38 %) and its presence was not influenced either by the natural region or by the altitude.

H. dermestoides was caught in 28 traps (presence rate of min. 16 %). Even if not as common as the bark and ambrosia beetles caught, it is present in a large part of Wallonia (Fig. 7). It should be noticed that no *H. dermestoides* were captured in the "Loess" and "Sandy loam" regions : however, the low number of traps in those regions is the reason for this apparent absence. Indeed, in 2002, we observed in the "forêt de Soignes" (a 4,000 ha beech stand located in the sandy loam region) several adults and many typical larval galleries on beech logs and snags.

DISCUSSION AND CONCLUSION

The omnipresence of *T. domesticum*, *T. signatum* and *X. dispar* in Wallonia was to be expected, considering the characteristics (particularly climate and breeding substrate requirements, as well as European distribution) of

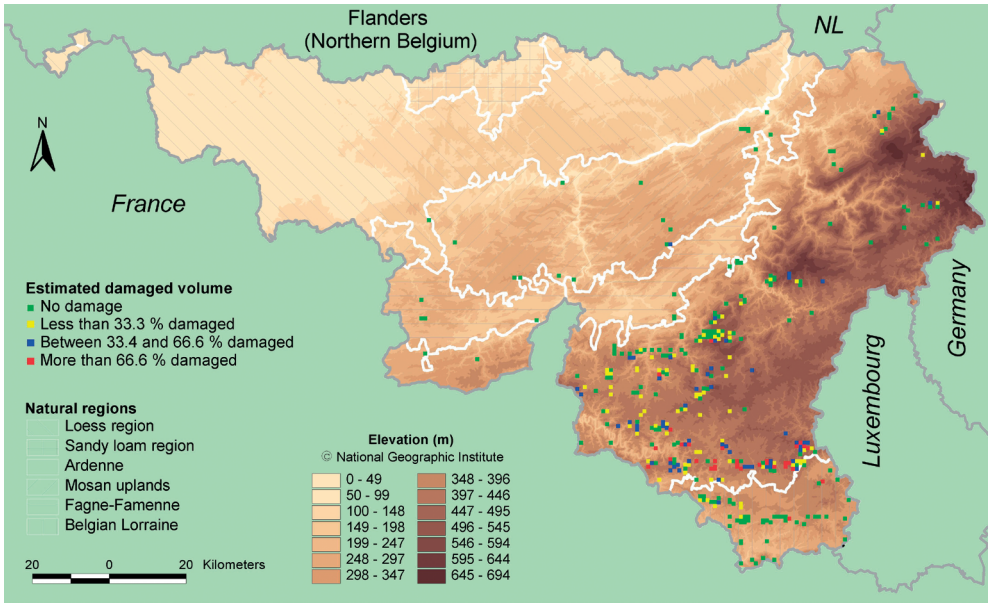


Fig. 1. – Distribution of damaged beech stands in Wallonia, according to a specific survey carried out i 2001 (381 sampling plots), in the context of an unusual beech disease (Rondeux et al., 2002).

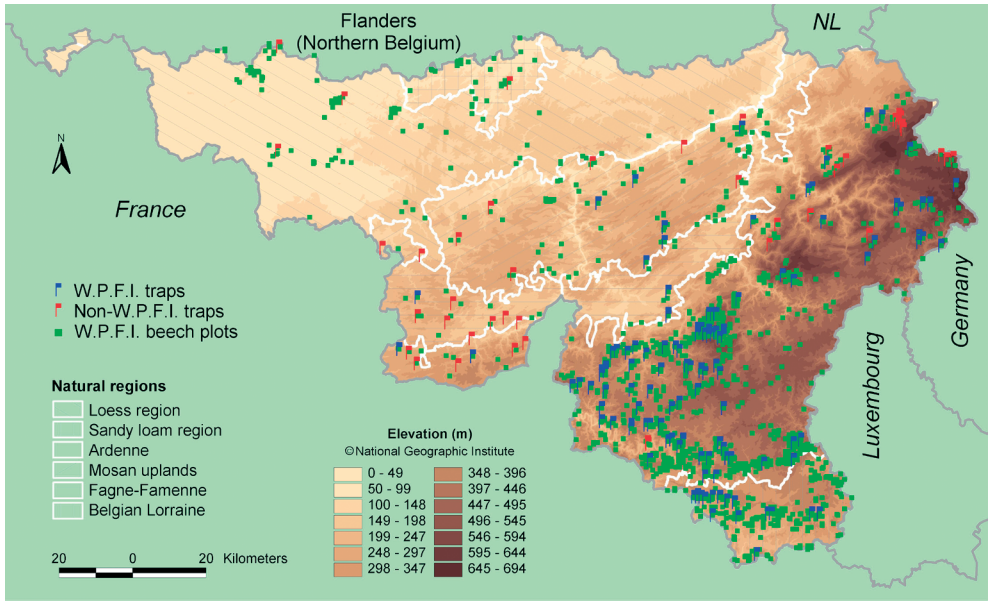


Fig. 2. – Trap network and beech plots (relative G_{beech} > 50%) belonging to the Walloon Permanent Forest Inventory (W.P.F.I.) plot network

those indigenous species (see e.g. Balachowsky, 1949). According to Decelle (1995), *T. domesticum* and *T. signatum* are also quite common in the forêt de Soignes : personal observations made in 2002 confirmed this fact. Dourojeanni (1971) also reported a wide distribution of these three ambrosia beetle species in Wallonia. Taking into account the variability of the number of traps in the natural regions, the observed presence rates of the three species do not seem to vary between the six Walloon natural regions (Henin et al., 2003). Concerning *T. bicolor*, Dourojeanni (1971) reported only two observations for Wallonia. In 1995, Decelle mentions a third observation in the forêt de Soignes. The much wider distribution we found seems however logical, considering the omnipresence of beech stands in Wallonia and the ecological niche[3] occupied by *T. bicolor* (see e.g. Balachowsky, 1949; Nicolai, 1997; Dajoz, 1998). Up-to-date data on this species are important as it seems to be involved in beech decline (Kohnle et al., 1987; Nageleisen, 1994) and able to cause damage on living trees (Schönherr & Krautwurst, 1979). Finally, *H. dermestoides* has long been considered as extremely rare in our Region (Collart, 1952). Our survey evidenced that, as expected by

[3] As defined by Hutchinson (1957, in Begon et al., 1996).

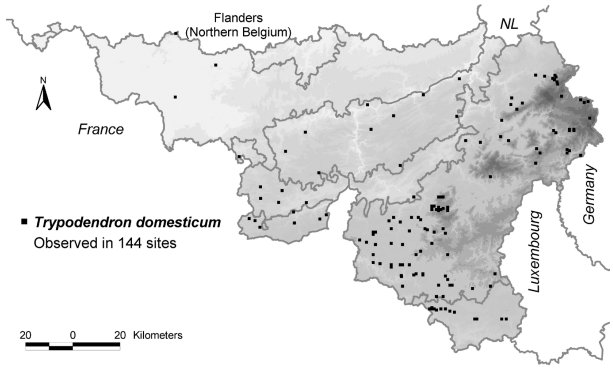
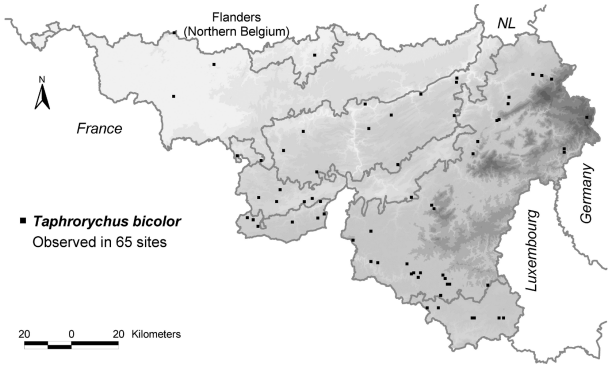


Fig. 3



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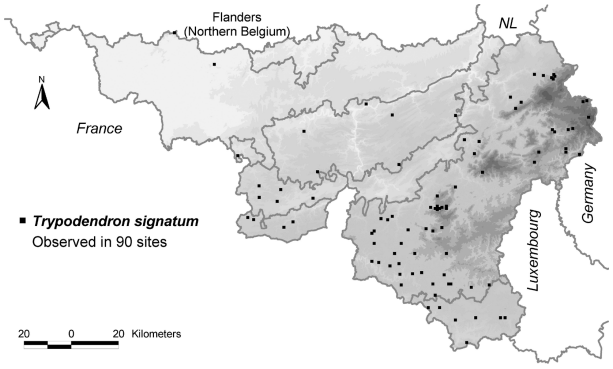

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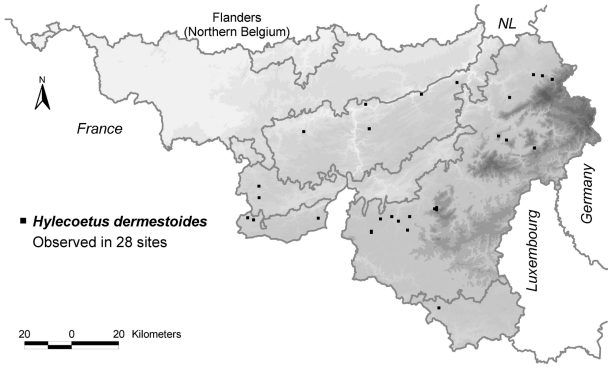

Fig. 7

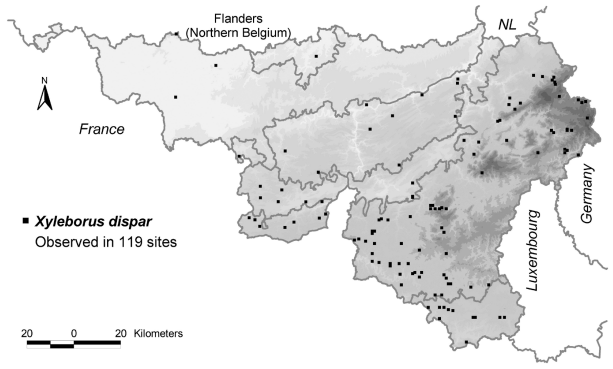

Fig. 5

Figs 3-7. – Distribution of five beech damaging Coleoptera in Wallonia, based on a 172 traps survey network set up in 2001.

the same author, this opinion is biased : in Wallonia, this coleopteran is widespread and may be locally extremely abundant (pers. obs.). Like COLLART (1952), we think that the short flight period and climatic requirements for flight explain the scarcity of this species in Belgian entomological collections. Because of its abundance and of the damage it may cause on many tree species (e.g. beech, oak and spruce trees), VRIJDAGH (1952) considers that *H. dermestoides* may locally have a pest status. Besides, in 2001 it also caused considerable damage in many beech stands (both on logs and weakened trees).

The observations here presented were made during a period of high and widespread availability of weakened beech trees. The five studied insects are favoured by those conditions : besides, the populations of *T. domesticum* and *T. signatum* reached epidemic levels over a wide area. It could be argued that this abnormal abundance of suitable hosts could have influenced our observations and the resulting maps. However, we think it reasonable to assume that these maps also reflect the beetles' distribution in non-epidemic conditions. Bark and ambrosia beetles (as well as *H. dermestoides*) are indeed dependent on ephemeral and generally scattered breeding substrate (EIDMANN, 1985; FORSSE & SOLBRECK, 1985; SCHIEGG & SUTER, 2000). Well adapted to seek out this resource, most of the scolytids are strong flyers, often able to spread over several, sometimes tens of kilometres (ATKINS, 1961; NUORTEVA & NUORTEVA, 1968; BOTTERWEG, 1982; NILSSEN, 1984; FORSSE & SOLBRECK, 1985; JACTEL & GAILLARD, 1991; BYERS, 2000). More specifically, DYER (1961) and CHAPMAN (1958, in DYER, 1961) report "considerable flight potential" in the genus *Trypodendron*. The first author suggests that *Trypodendron lineatum* (Olivier, 1795) "may, in some circumstances fly several miles to attack logs". Thus, it is likely that Wallonia is permanently inhabited with variable local population levels of the studied Coleoptera. Once the availability of weakened trees increases locally, pre-existing populations grow and may reach epidemic levels : it is assumed that a part of the population behaves rather sedentarily, while another part spreads to neighbouring or relatively remote areas, possibly anemochorously (migration being an important process in the dynamics of most bark beetle populations, according to FORSSE & SOLBRECK, 1985; DUELLI et al., 1997). Hence, the major part, if not the

totality of the small region that is Wallonia is very likely permanently submitted to the action of these indigenous insects. We agree with Beaver & Löyttyniemi (1991) who, although working on Zambian bark and ambrosia beetles, suggest a stable distribution over time on a regional scale, "even though the species involved are opportunistic colonists of temporary habitats which, in natural conditions, are likely to show a strongly heterogeneous distribution in space and time on a local scale".

The omnipresence of both studied *Trypodendron* spp. in Wallonia on the one hand, and the occurrence of damage only in the Ardennes and Belgian Lorraine on the other, is evidence of the lower population levels in the other Walloon natural regions (assuming that, in stands under similar sanitary conditions, the higher the population the heavier the damage), and/or of the strict location of the trigger factor in both natural areas. These facts confirm, to some extent, the secondary pest status[4] of both involved ambrosia beetles. Furthermore, careful observations and experiments realised during the summer of 2001 proved that *T. domesticum* and *T. signatum*, unlike what was feared in the spring of the same year, are definitely secondary pests (Grégoire & De Proft, unpubl.). Indeed, although looking healthy, the trees attacked in 2001 exhibited cortical micro-necrosis and/or were covered with dying mosses : although not yet understood, the relationship between those elements and the entomological attacks appeared strong (Huart et al., 2003). Consequently, the pest management program (that was based upon phytosanitary cuttings, the set up of trap trees and the use of 20,000 flight barrier traps baited with ethanol and lineatin) has been set up only in the Ardennes and Belgian Lorraine. The other natural regions of Wallonia, although inhabited by *T. domesticum* and *T. signatum*, do not seem, in the context of the persistence of the 2000-2001 crisis, to be in danger of unusual large-scale beech attacks by those secondary pests.

From a wider point of view, these results highlighted the lack of "basic knowledge" about the tiny, unattractive insects we have studied. However, although more common in resinous stands, large-scale scolytid outbreaks of economic importance have already occurred in Belgian deciduous forests, notably in beech stands. Sixty years ago, for several years after the hard winter of 1942, *T. domesticum* caused extensive damage to beech trees in the Ardennes (Prieels, 1954; Poncelet, 1965). It is likely that *T. signatum* was also involved in this damage, but owing to its relatively lower abundance in central Europe compared with *T. domesticum* (e.g. Paiva, 1982; Payne et al., 1983; pers. obs.), and close resemblance to the latter, the former was not detected. Besides, although indigenous, *T. signatum* was first recorded in Belgium by Debatisse only in 1945 (Dourojeanni, 1971). Nevertheless, and despite the known pest-status of both species, the Walloon Nature and Forest Division (W.N.F.D.) lacked up-to-date biogeographical data concerning these disregarded insects. The forest managers and phytopathologists had little information (based on randomly collected data), scattered in different organisations and not always easily accessible. This unfortunate situation could be improved, to some extent, by permanent monitoring of the most important forest pest species and tree pathologies. From this viewpoint, a part of the W.P.F.I. plot network could be devoted to the collection of entomological and phytosanitary data. Each plot of this network being accurately characterised in terms of forest structure, productivity, edaphic conditions, topography, phytosociology, etc., it would also be an efficient tool to assess and understand the relationships between those factors and pest population dynamics or phytosanitary problems.

[4] A pest is secondary when its development is dependent on physiological deficiency of its host. Oppositely, the insects able to develop in healthy, not physiologically weakened trees are called primary pests (Rudinsky, 1962).

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Paradicranophorus sinus sp. nov. (Dicranophoridae, Monogononta) a new rotifer from Belgium, with remarks on some other species of the genus *Paradicranophorus* Wiszniewski, 1929 and description of *Donneria* gen. nov.

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ABSTRACT. A new dicranophorid rotifer, *Paradicranophorus sinus* sp. nov., is described from periphyton originating from poikilohaline waters in Belgium. The main distinguishing taxonomic features of the new species are intramallei with supramanubria and a pair of preuncinal teeth. Taxonomic problems associated with the genus *Paradicranophorus* are briefly discussed. A new genus, *Donneria*, is proposed to accommodate *Paradicranophorus sudzukii* Donner, 1968. Information is presented on the trophi of *P. hudsoni* (Glascott, 1893) and *D. sudzukii* (Donner, 1968). *Encentrum brevifulcrum* Dartnall, 1997 is synonymised with *P. sordidus* Donner, 1968.

KEY WORDS : Rotifera, taxonomy, *Paradicranophorus*, new species, *Donneria* gen. nov., Dicranophoridae, thalassic waters, Belgium.

INTRODUCTION

Despite the recent upsurge of interest in rotifer biology and taxonomy, our knowledge of brackish and marine species still remains in its infancy world-wide. Information on brackish and marine rotifers from Belgium is largely restricted to plankton studies and opportunistic species records (see overviews in De Ridder, 1961, 1989, 1992; De Smet, 1994, 1997, 2000). Virtually nothing is known about the species inhabiting psammic and periphytic habitats. In this contribution a new species of *Paradicranophorus* discovered among periphyton is described, and information on some other species belonging to the genus is presented. The status of the genus *Paradicranophorus* is discussed, and a new genus is established.

MATERIAL AND METHODS

The species was collected from algae during an ongoing study on the rotifer fauna of littoral thalassic habitats of the Belgian coast. Both samples for live examination and samples fixed with formaldehyde to a final concentration of 4 %, were taken. The rotifers were extracted in the laboratory using the swirl-decantation technique, and consequently concentrated on a 40 µm sieve. Animals were examined and drawn using a Leitz Orthoplan microscope fitted with a camera lucida. Preparation of trophi for light and scanning electron microscopy (S.E.M.) was done following DE SMET (1998) using NaOCl solution. For S.E.M. a Philips S.E.M. 515 operated at 20 kV was used.

RESULTS

Paradicranophorus sinus sp. nov.
(Figs 1-12)

Type locality. Ditch in Zwin Nature Reserve, Knokke Belgium. 22 April 1996.

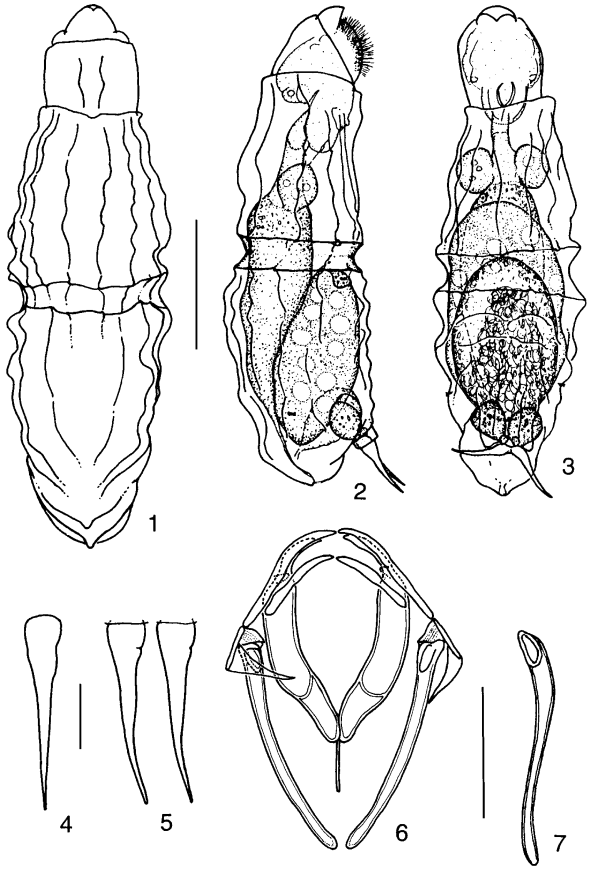
Material. Holotype : a female in a permanent, glycerine glass slide mount deposited in the Koninklijk Belgisch Instituut voor Natuurwetenschappen (K.B.I.N.), Brussels, Belgium, N° IG 29.287.

Paratypes : 62 females from type locality; data the same as for holotype. One female and trophi preparation in the K.B.I.N. One female and trophi preparation in the Laboratory for Animal Ecology, University of Gent, Belgium. Ten mounted paratypes, three light microscopy preparations and five S.E.M. trophi preparations in R.U.C.A.

Additional material : two females collected in the littoral of the Western Scheldt, St. Annastrand, Antwerpen, Belgium, 18 November 1995.

Etymology. The species name *sinus*, *-us* is the Latin noun for creek, bay, and refers to the Middle Dutch meaning of the name Swin of the type locality.

Diagnosis. The provisional generic placement of *P. sinus* sp. nov. is based on the following combination of character states : the overall shape of the body, the presence of transversal furrows in the trunk, the ventrally-displaced foot, and the shape of the elongate trophi. The new species is diagnosed by the presence of (1) 1-2 deep circular transversal



Figs 1-7. – *Paradicranophorus sinus* sp. nov. 1. dorsal view, 2. lateral view, 3. ventral view, 4. toe, dorsal view, 5. toe, lateral view, 6. trophi, ventral view, 7. manubrium, lateral view. Scale bars : 1-3 : 50 µm, 4-7 : 10 µm.

folds in the trunk, (2) intramallei and (3) a pair of preuncinal teeth.

Description

Parthenogenetic female. Body fusiform in dorsal view, broadly rounded or obtusely acute posteriorly; in lateral view highest in posterior half, abdomen bulging behind last foot pseudosegment, rounded. Head offset by distinct neckfold. Rostrum conspicuous, broad triangular, decurved ventrally. Submentum indistinct. Ventral papillae absent. Dorsal antenna small, in shallow depression near middle of head. Corona small, oblique. Trunk with longitudinal and transversal folds; 1-2 deep circular transversal furrows near mid-length; 1-2 V-shaped folds postero-dorsally. Cuticle weakly sticky, covered with detritus. Lateral antennae comparatively small, in posterior third of trunk. Foot ventral, two pseudosegments; large basal pseudosegment continuous with trunk, forming bulging section behind small distal pseudosegment; anterior delimitation of distal pseudosegment unclear. Toes swollen at their base, tapering towards acute apices, in dorsal view straight, in lateral view slightly decurved ventrally near posterior third or slightly S-shaped.

Eyespots absent. Brain saccate, short, with Y-shaped duct. Subcerebral glands large, with light-refracting globule. Salivary glands in mastax. Proventriculus absent. Gastric glands large, rounded, very short-stalked. Two large and two small ovate pedal glands. Bladder normal. Vitellarium with ten nuclei. Subitaneous egg (inside body) ovate. It was impossible to determine whether the new species is viviparous or not.

Trophi forcipate, elongate, simple (Figs 6, 7, 8-12). Outline of rami elliptical; outer margin of rami slightly blunt-angular near transition of basal and sub-basal chambers; inner margin of rami without teeth. Median rami opening elongate drop-shaped; rami almost parallel-sided the greatest part of their length; each ramus with single, stout and distinctly offset apical tooth, set at an oblique angle to the trophi axis; opening of sub-basal chambers distally, small, rounded; opening of basal chambers dorsally, small, rounded, at posterior 1/3 of rami. Fulcrum very short, c. 1/4 ramus length, in lateral view plate-shaped, broad, rounded posteriorly. Unci single-toothed, long; shaft slightly longer than tooth; dorsal apophysis present only, conspicuous, triangular; lateral ribs of shaft weak, most pronounced postero-ventrally; uncinal teeth weakly curved. Each ramus with single preuncinal tooth; preuncinal teeth almost straight, long, c. 2/3 uncus length. Epipharyngeal fans not observed. Intramallei plate-shaped, curved, elongate-triangular, anterior half connected to unci and dorsal triangular expansion of head of manubria respectively; the latter connection by a series of c. ten fairly long and appressed ligaments; posterior half free, pointing latero-distally, bearing supramanubria. Supramanubria elongate-triangular, ending in more or less filamentous part inwardly, outer part of supramanubria grasping inner part of intramallei both anteriorly and posteriorly. Manubria long, c. incus length, very slightly curved in dorsal view, very slightly undulate in lateral view; head with triangular ventral and dorsal expansions.

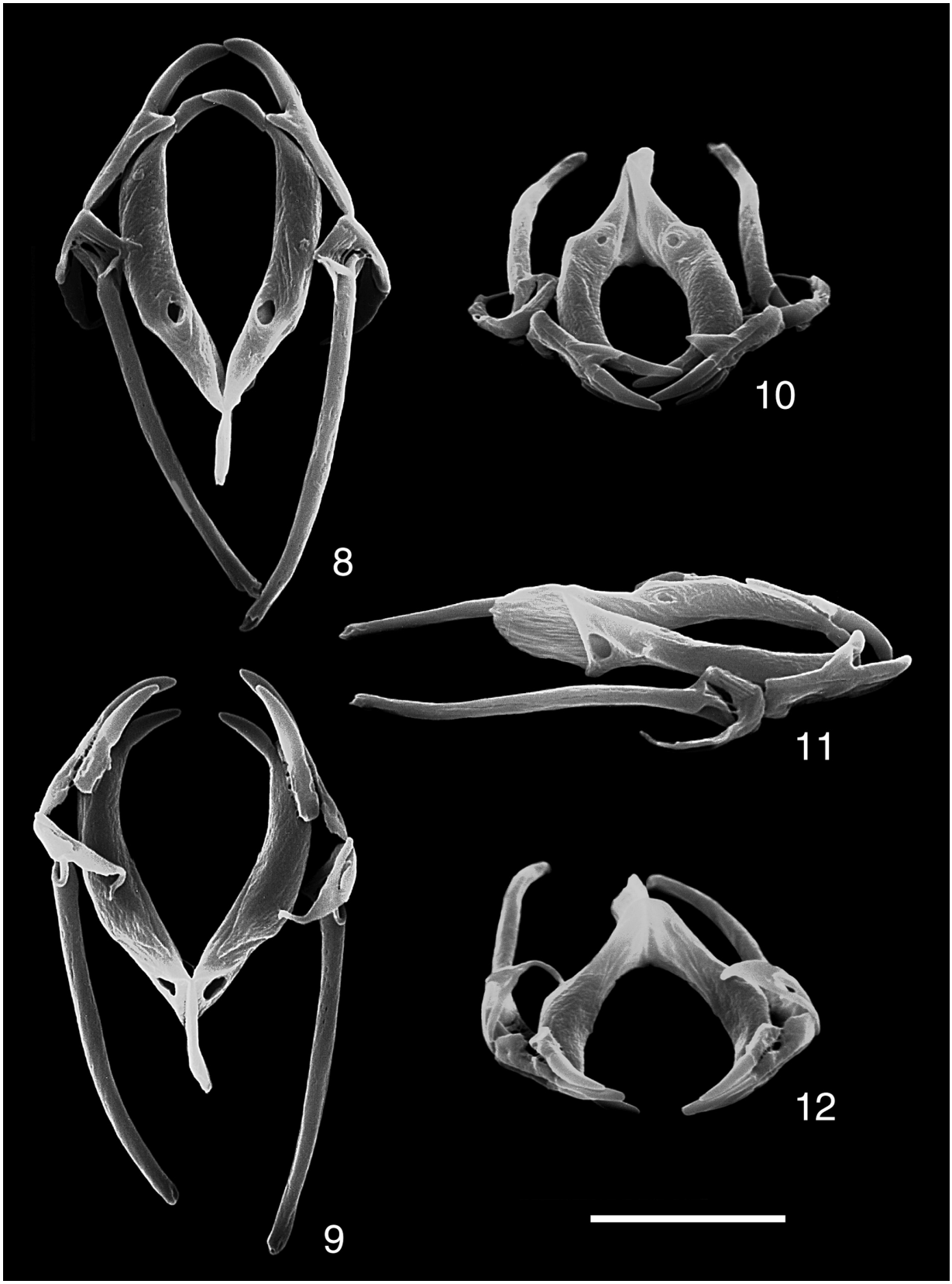
Male unknown.

Measurements. N=14. Total length 165-205 µm, toe 22-30 µm. Trophi 22.3-28.6 µm : ramus 11.4-16.6 µm, fulcrum 2.9-4.4 µm, uncus 7.4-10.5 µm, preuncinal tooth 5.1-7.0 µm, intramalleus 4.4-5.2 µm, supramanubrium 5.7-6.6 µm, manubrium 16.3-19.0 µm. Subitaneous egg (inside body) 76x46 µm.

Comments

Five species of *Paradicranophorus* were recognized in a recent revision by De Smet (1997) : *P. aculeatus* (Neiswestnova-Shadina, 1935), *P. hudsoni* (Glascott, 1893), *P. sordidus* Donner, 1968, *P. sudzukii* Donner, 1968 and *P. verae* Bogoslovsky, 1958. A sixth species, *P. wesenberglundi*, assigned to the genus was recently described by Sørensen (2001). The different species can be discriminated unequivocally by their trophi structure.

P. sinus sp. nov. is easily distinguished from *P. sudzukii* (*Donneria sudzukii* comb. nov., see further) by its elongate rami, which are semi-circular in the latter species. It differs from *P. aculeatus*, *P. hudsoni*, and *P. verae* by the presence of intramallei, which are absent in the latter. The new species shares the characters, presence of intramallei and supramanubria, with *P. sordidus* and *P. wesenberglundi*. However, *P. sordidus* exhibits two preuncinal teeth prior to each apical ramus tooth instead of a single one. *P. wesenberglundi* likewise displays a single preuncinal tooth, but the outer margins of its rami are angular



Figs 8-12. – *Paradicranophorus sinus* sp. nov., SEM photographs of trophi. 8. dorsal view, 9. ventral view, 10. dorso-apical view, 11. dorso-lateral view, 12. ventro-apical view. Scale bar : 10 µm.

postero-laterally, instead of gradually incurving towards the fulcrum.

On the basis of external morphology alone, the new taxon could be confused with juvenile forms of *P. hudsoni.*

Distribution and ecology

To date, *P. sinus* sp. nov. is only known from its type locality, the Zwin (Knokke) and St. Annastrand, River Western Scheldt (Antwerp). It was collected in the littoral zone among the green alga *Enteromorpha intestinalis* (L.) Link and tufts of the substrate-bound filament-forming colonial diatoms *Parlibellus delognei* (Van Heurck) Cox and *P. pseudocomoides* (Hendey) Cox in the Zwin, and among the mat-forming siphon alga *Vaucheria* sp. in the Scirpo-Phragmitetum from the littoral of the river Scheldt. It thus can be assumed that *P. sinus* sp. nov. is a periphytic and littoral species. The chloride content of the Zwin water is highly variable, and fluctuates between mesohaline (1-10 g $Cl^{-}l^{-1}$) and sea-water (>17 g $Cl^{-}l^{-1}$) depending on the tides of the North Sea, evaporation, and dilution by freshwater (rain, supply from inland). A chloride content of 18 g $Cl^{-}l^{-1}$was measured at the time of sampling. The Scheldt estuary is characterized by a deeply-penetrating tidal wave, creating an inland salt gradient. At St. Annastrand the water of the Scheldt ranges from fresh to mesohaline water. It follows that *P. sinus* sp. nov. is an euryhaline species inhabiting poikilohaline waters. The finds of the species in April and November suggest that it prefers low temperatures.

Analysis of the gut-content showed that *P. sinus* sp. nov. feeds on diatoms.

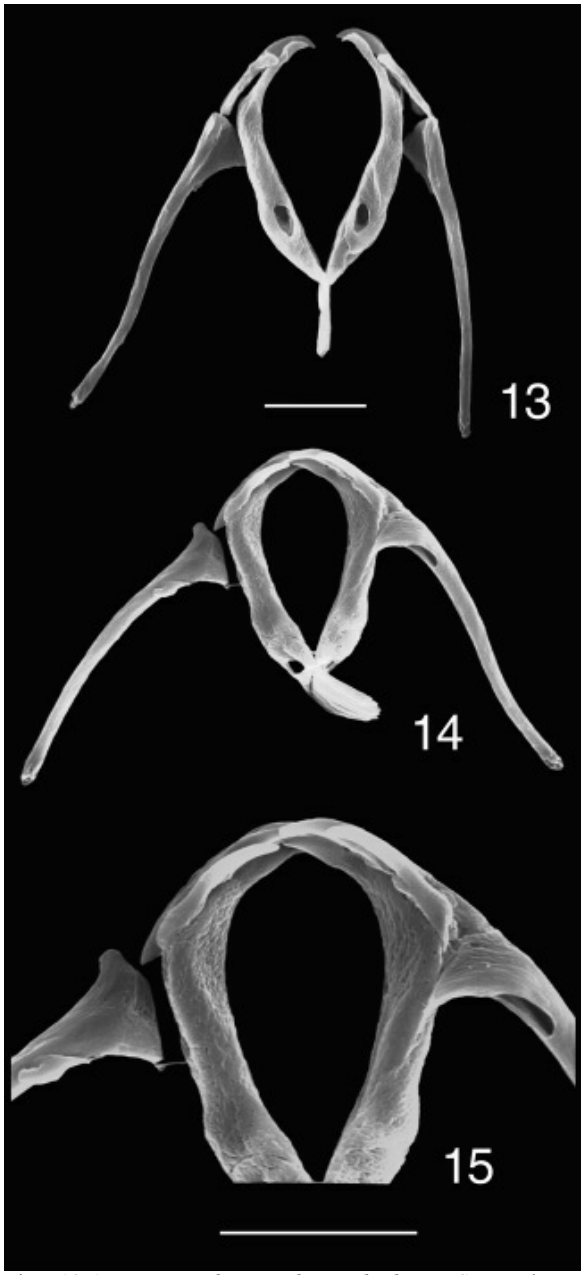
The accompanying rotifer fauna at the type locality consisted of unidentified bdelloids and the monogononts *Proales reinhardti* (Ehrenberg, 1834), *Encentrum algente* Harring, 1921, *E. marinum* (Dujardin, 1841) and *E. obesum* Tzschaschel, 1979. At St. Annastrand *P. sinus* sp. nov. co-occurred with *Cephalodella gibba* (Ehrenberg, 1832), *Encentrum algente*, *E. glaucum* Wulfert, 1936, *E. limicola* Otto, 1936 and *Encentrum* sp.

Remarks on other *Paradicranophorus* spp.

The study of additional material of some *Paradicranophorus* species allows for corrections and new information since the review by De Smet (1997).

P. hudsoni
(Figs 13-15)

SEM of the trophi reveals that the so-called subunci are in fact preuncinal teeth (according to nomenclature used in dicranophorids), participating in the formation of the ramus lock. The rostrum is broadly triangular and not rounded. To date, this probably cosmopolitan species (Koste & Poltz, 1983; De Smet, 1997) has been recorded in Belgium from benthos of the shallow (depth 1 m) Blokkersdijk, Antwerpen only (De Smet, 1997). I have now found it also during the cold season in benthos of the Put van Ekeren, Antwerpen to a depth of 8 m.



Figs 13-15. – *Paradicranophorus hudsoni*, SEM photographs of trophi. 13. dorsal view, 14. ventral view, 15. ibidem, detail. Scale bars : 10 µm.

P. sordidus

Examination of the type material of *Encentrum brevifulcrum,* described by Dartnall (1997) from fresh and slightly saline lakes in the Vestfold Hills, eastern Antarctica, shows that it is synonymous with *P. sordidus.* Instead of rami "crowned by two small teeth" (Dartnall, 1997 : 15, Fig. 2a), each ramus exhibits a single apical ramus tooth and two preuncinal teeth as shown by *P. sordidus*. Up to now *P. sordidus* was only known from its type locality Neusiedler See, Austria (Donner, 1968; Koste & Zhuge, 1995), and Blokkersdijk, Antwerpen, Belgium (De Smet, 1997).

P. sudzukii
(Figs 16-24)

The trophi of *P. sudzukii* have been studied to date by light microscopy only (Donner, 1968). Scanning electron microscopy reveals some interesting features of the preuncinal teeth, intramallei, supramanubria, manubria and unci. The apical rami teeth mentioned by Donner (l.c.) actually prove to be well-developed preuncinal teeth (Fig. 17), extending far beyond the apical rami teeth, which are set at a more or less right angle to the trophi axis. Thus, the rami outline is roundish-depressed instead of rounded-elliptical. The strongly inwardly bent dorsal teeth at the inner margins of the rami prior to the acute tip of Donner (l.c.) prove to be the apical rami teeth. The dorsal placement of these teeth was questioned by De Smet (1997), who interpreted them as preuncinal teeth, based on Donner's misinterpretation of the apical rami teeth. Indeed, the ventrally-located preuncinal teeth are usually set at an equal or less acute angle to the trophi axis than the apical rami teeth. Unique among the dicranophorids studied so far, is the partial fusion between the intramalleus and the head of the manubrium (Figs 17, 22, 23). This fusion mainly concerns the ventral and dorsal margins of both trophi elements. Instead of each having their own opening, intramallei and manubria show a common opening (co; Figs. 22). A small vestigial chamber is present on the ventral edge of the head of the manubria. The supramanubria (s; Fig. 22) deviate from the usual single-element configuration, in being composed of two basally-fused sclerite elements, each with free elongate projection pointing inwardly. Donner (l.c., p. 225, Fig. 1.f) did not mention intramallei or supramanubria, but indicated the posterior margin of the supramanubria in his figure of the trophi. The unci are two-toothed (Fig. 24) instead of single-toothed. The shortest dorsal tooth is incurved anteriorly and functions as uncinal apophysis. Its incurved part is supported by a small process or uncinal apophysis of the greatest tooth (Fig. 21). Uncinal seams are lacking. Large and stout epipharyngeal fans are distinct (ep; e.g. Figs 17, 20).

Measurements. (N=2). Total length (slightly contracted) 230 µm, toe 28-30 µm, trophi 25-30 µm, ramus 9-10 µm, fulcrum 9-10 µm, uncus 11-12 µm, subuncus 7-7.5 µm, intramalleus 5-5.5 µm, supramanubrium 7.6-7.7 µm, manubrium 18-19 µm.

The specimens used in the present study came from psammon of the Colorado river, Colorado, U.S.A. To date *P. sudzukii* was only known from benthos of its type locality Neusiedler See, Austria.

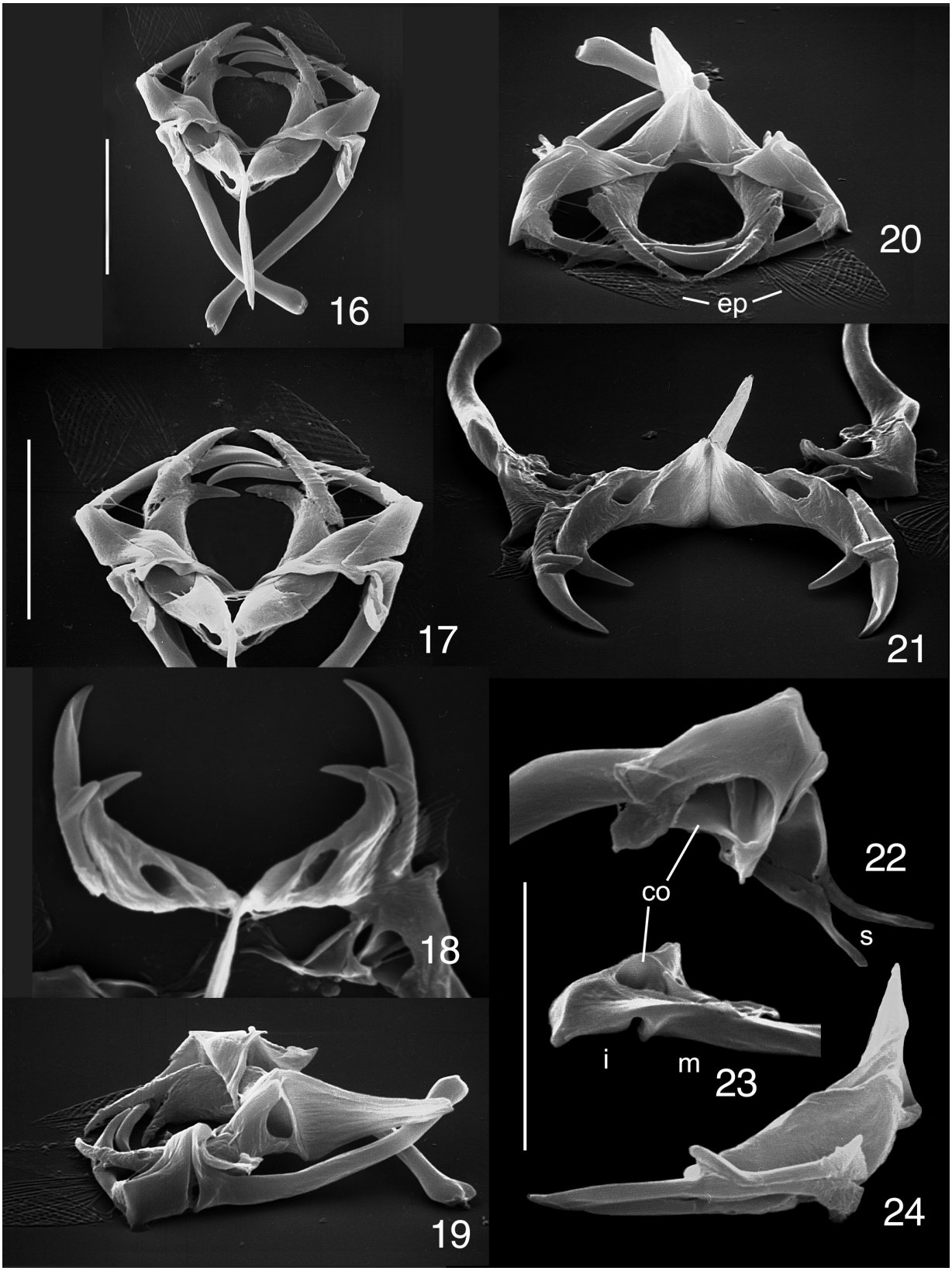
Remarks on the genus *Paradicranophorus*

The type species of the genus, *P. hudsoni*, was originally described sub *Diglena hudsoni* by Glascott (1893), and later transferred to *Dicranophorus* by De Beauchamp (1929), who also suggested that a special genus would not be out of place in view of its individuality. Uninformed of the papers by Glascott (l.c.) and De Beauchamp (l.c.), Wiszniewski (1929, 1931/32) described the same species and introduced the generic name *Paradicranophorus* for species of the Dicranophoridae (at that time a subfamily of the Notommatidae) having : a pyriform body with characteristic furrows, a small and ventrally-displaced foot with two short toes, an almost ventral corona composed of two ciliary fields without locomotory capacity, and lyrate forcipate trophi, to mention the most important characters. Until recently (Koste, 1978, 1985; Koste & Zhuge, 1995) the genus currently comprised six described species. De Smet (1997) recognized five species (*P. aculeatus*, *P. hudsoni*, *P. sordidus*, *P. sudzukii*, *P. verae*) and assigned *P. wockei* to the new genus *Kostea* on basis of the notable differences in morphology of the body and trophi. The new data on trophi morphology presented above allow a more detailed comparison of the species. An overview of the characters traditionally considered of taxonomical significance are briefly discussed below.

The body outline varies from fusiform (*P. aculeatus*, *P. sudzukii*, *P. wesenberglundi*) to pyriform (*P. hudsoni*, *P. verae*) with transitions between (*P. sinus*, *P. sordidus*). Consequently body form only helps to a certain extent in distinguishing the species, and is of little value in discriminating the genus from other dicranophorid genera. The pyriform shape of the body is probably an adaptation to the special environment, i.e. the surface of loose mud, in which most of the species live. At least in *P. hudsoni* it helps in anchoring, when during feeding it rotates all around the anterior part of the body prior to the circular transversal fold (pers. obs.). *P. wesenberglundi* has a well-developed corona allowing the species to swim in the water column (Sørensen, 2001). The other species display a weakly-formed corona resulting in a reduced swimming ability, and creeping way of locomotion using peristaltic movements (*P. aculeatus*, *P. hudsoni*, *P. sinus, P. sudzukii*) and/or snake-like wiggling of the body (*P. aculeatus, P. sudzukii*). The pyriform body and the more or less deep transversal and longitudinal folds (reported in all species, *P. verae* excepted), probably are adaptations that may help in obtaining a grip when creeping. There is no consistent patterning of either longitudinal or transversal folds. Deep circular transversal furrows are shared by *P. hudsoni* and *P. sinus* only. It follows that the presence and patterning of folds can not be used as a general character of the genus. The ventral head papillae of *P. aculeatus* are unique structures in rotifers, that probably serve for anchoring in crevices of the sand system or in capillary areas of detritus deposits (Koste & Zhuge, 1995). A prominent submentum has been reported for the poorly-described *P. verae* only. The lateral antennae are situated in the anterior third of the body in *P. sudzukii*, and in the posterior third in the other *Paradicranophorus* spp. To our present knowledge, lateral antennae lying in the anterior part of the body have not been observed for any other dicranophorid species.

Two main types of trophi can be distinguished on basis of the rami outline and relative length of the trophi. In the first type, the rami are elongate and the fulcrum is relatively short (ratio length ramus : length fulcrum varying from 3.5 to 5.7); it is present in *P. aculeatus, P. hudsoni, P. sinus, P. sordidus, P. verae* and *P. wesenberglundi*. In the second trophi type, the rami outline is rounded and the fulcrum is relatively long (ratio length ramus : length fulcrum on average 1.0); it is found in *P. sudzukii*.

In the species of the first type with elongate rami and short fulcrum, some display preuncinal teeth (*P. hudsoni, P. sinus, P. sordidus, P. wesenberglundi*), which are reported missing in others (*P. aculeatus* (Pawlowski, 1956, 1958; Neiswestnowa-Shadina, 1935; Ovander,



Figs 16-24. – *Donneria sudzukii* comb. nov., SEM photographs of trophi. 16. ventral view, 17. as Fig. 16, detail, 18. incus and unci dorsal view, intramalleus and head of manubrium, inner view, 19. ventro-lateral view, 20. ventro-apical view, 21. dorso-apical view, 22. intramalleus, head of manubrium and supramanubrium, inner view, 23. intramalleus and head of manubrium, dorsal view, 24. uncus, lateral view. Scale bars : 10 µm.

1977; Koste & Zhuge, 1995), *P. verae* (Bogoslovsky, 1958)). Care should, however, be taken in interpreting this lack of preuncinal teeth, because it is my experience with the dicranophorid genus *Encentrum* that these elements were often overlooked when light microscopy was used. A probably phyletically more fundamental difference within the group displaying trophi of the first type, is the trait presence/absence of intramallei. Intramallei are present in *P. sinus*, *P. sordidus* and *P. wesenberglundi,* and absent in *P. aculeatus, P. hudsoni* and *P. verae.* Well-developed intramallei is one of the main diagnostic features in *Encentrum,* although similar but usually less-developed sclerite elements have been reported in *Erignatha*, *Dicranophorus* (two species considered *incertae sedis*), *Inflatana*, *Kostea*, *Wierzejskiella* and *Wigrella* (De Smet, 1997). However, the overall similarity of the trophi of intramallei-bearing *Paradicranophorus* spp. is greatest with the genus *Encentrum*, particularly with *E. uncinatum* and related species. Considering this and the above-mentioned diversity in characters of generic value (viz. body shape, corona, transversal folds, placement of foot, etc.), the actual genus *Paradicranophorus* (exclusive of *P. sudzukii*, see below) may include two distinct groups, namely (1) species of the first trophi type without intramallei, (2) species of the first trophi type with intramallei related to, or belonging to *Encentrum.* The placement of the new species into *Paradicranophorus* is therefore provisional, pending new information by molecular techniques.

The species displaying the second trophi type with roundish-depressed rami outline and long fulcrum (*P. sudzukii*), moreover differs considerably from the other *Paradicranophorus* species in the dimension and position of the preuncinal teeth, the shape and structure of the malleus, in particular the partially-fused intramallei and manubria with common opening, the supramanubria composed of two sclerite elements, and the large and stout epipharyngeal fans. This character state, in combination with the lateral antennae located in the anterior third of the body, likewise distinguishes *P. sudzukii* from the other dicranophorid genera, and in my opinion provides sufficient justification for a distinct generic status.

Donneria gen. nov.

Diagnosis. Body fusiform, foot placed ventrally. Lateral antennae in anterior third of trunk. Trophi forcipate, with partially-fused manubria and intramallei, showing common opening. Supramanubria composed of two basally-fused sclerite elements, each with free elongate projection. Rami outline roundish. Preuncinal teeth long, projecting beyond apical rami teeth. Unci consist of two appressed teeth. Fulcrum long, ramus length.

Type species. *Paradicranophorus sudzukii* Donner, 1968, by present designation.

Etymology. The genus is named in honour of the late Josef Donner (1909-1989), eminent Austrian rotiferologist.

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SHORT NOTES

Prey selection patterns in *Notonecta maculata* Fabricius, 1794 (Insecta, Hemiptera)

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Notonecta maculata Fabricius, 1794 is an aquatic species of Hemiptera widely found in the Western Palearctic region (5), where it can be considered a key player as a secondary consumer (1) principally of still water systems. Thus, it is specially interesting to increase our knowledge of the trophic ecology of this species, which is considered an opportunist (4) feeding mainly in natural pools, on terrestrial arthropods that fall on the water surface (6). The role of this aquatic bug in the predation of animals trapped on water surfaces has been analysed in few studies (2).

The aim of the present experimental study was to detect the possible prey selection patterns of *N. maculata* at the water surface in relation to type, state (alive or dead) and size of prey. The response of the species to different vibration frequencies was also studied.

The study was conducted in an artificial pond in Alfacar (S Spain) in early spring. The *N. maculata* population in the study pond was very high; in excess of one thousand exemplars. All the experiments were carried out between 16:00 and 18:00 sun time (water temperature 18-21ºC). Potential prey were thrown into different areas of the pond, the existence or not of a response was detected, and response time was measured. Three prey types : bee (*Apis mellifera*), ant (*Messor* sp.) and grasshopper (*Oedipoda* sp.) were selected. For each prey type, 15 alive and 15 dead individuals (killed by freezing to avoid the use of odourous substances) were randomly thrown into the water. Previously, all the individuals were measured (precision ± 0.01 cm). As control, blades of grass, measured previously and thrown the same number of times (n = 15), were employed. It was considered as an affirmative response when, in the first ten minutes, *N. maculata* actively contacted the prey, and response time was the time elapsed from the contact of the prey with water to the contact between prey and *N. maculata*. To study the response time to different vibration frequencies, a mechanical vibrator model SF-9324 Pasco Scientific was employed (contacting with the water surface), using three frequencies (l, 5 and 10 Hz). Also, the response time was quantified in each experiment (15 times for every frequency and in different pond places).

For the three prey species, there was a higher response percentage for live individuals (100% for the three species) *vs*. dead ones (60%, 80% and 73.3% in ant, bee and grasshopper respectively), statistically significant for ant (χ^2= 7.5; df= 1; p= 0.006) and grasshopper (χ^2= 4.6; df= 1; p= 0.032) but not for bee (χ^2= 3.33; df= 1; p= 0.068). In the control, the response percentage was even less (40%; χ^2= 12.86; df= 1; p= 0.003). These results support the idea than *N. maculata* behaves, at the water surface, more as a predator than as a necrophage, particularly as an ambush predator (7). Moreover, these results indicate that the prey mobility, and not only the water perturbation derived from the contact after the fall, is related to the detection by *N. maculata,* as (3) noted for *N. glauca.* The lower response percentage to the control than to the dead prey indicates that *N. maculata* has the capacity to distinguish shape, colour or smell of the prey. In the present study, the opportunism of *N. maculata* was supported because it did not show any kind of selectivity between the three live preys offered, despite the clear differences in size, shape and movement type presented by each one.

The mean response time was always shorter with live prey : ant (29.2 s; SD = 44.1; N = 15), bee (40.5 s; SD = 42.7; N = 15) and grasshopper (67.9 s; SD = 90.3; N = 15) compared with dead ones: ant (120.0 s, SD = 136.0; N = 9), bee (104.2 s, SD = 108.4; N = 12) and grasshopper (110.4 s; SD = 125.9; N = 11). By means of a Man-Withney U-test analysis, we proved that the response time was significantly lower to live prey than dead prey (ant : Z= 2.48; p= 0.013; n1= 12; n2= 17; bee : Z= 1.96; p= 0.05; n1= 20; n2= 18; grasshopper : Z= 2.06; p= 0.04; n1= 17; n2= 15). These results, together with the previous ones, show that *N. maculata* not only respond more to the live prey but they do so more quickly. Moreover, these data show in relation to the time response an ant-bee-grasshopper gradation when the prey is live and bee-grasshopper-ant when dead. These results, in the case of live prey, seem to show a fast first selection for smaller prey (ant), perhaps because the bigger ones may present a risk or high energetic cost (grasshopper). In relation to the dead prey, *N. maculata* may have responded more to the heavier ones because they represent greater food quantity. Although a grasshopper is of greater length than a bee (approximately x 1.5 in the species used here), the total volume of the bee is relatively similar to that of the grasshopper, consistent with the similarity in selection of dead individuals of these two species by *N. maculata*. The analysis results show that there is no statistically significant correlation between individual prey size (within the same kind of prey) and response time (Table 1), perhaps

because the differences in size within one prey kind were not large.

TABLE 1

Pearson correlation coefficient
between prey size and response time

Prey	Live			Dead		
	r	p	n	r	p	n
Ant	0.60	>0.05	15	-0.18	>0.05	9
Bee	0.34	>0.05	15	0.42	>0.05	12
Grasshopper	0.33	>0.05	15	-0.41	>0.05	11

The results of the vibration experiments show a minor response percentage to low frequencies (53.3% of response to frequency 1, in comparison with 100% to frequencies 5 and 10) and, moreover, a minor response time to high frequencies ($\bar{x}$= 81.10 s, SD = 44.43, n = 8 for frequency 1; X = 28.3 s, SD = 35.66, n = 15 for frequency 5; $\bar{x}$ = 11.92 s, SD = 13.78, n = 15 for frequency 10). Man-Withney U-test analysis proved that the response time is significantly higher to frequency 1 than to the remaining frequencies (between 1 and 5 : Z= 2.84; p= 0.0045; n1= 8; n2= 15; between frequencies 1 and 10 : Z= 3.61; p= 0.0003; n1= 8; n2= 15). The differences between the mean response times for frequencies 5 and 10 Hz are not significant, but a tendency is observed (Z= 1.89; p= 0.059; n1= 15; n2= 15). These data show that the vibration frequency produced by a possible prey play an important role in the detection by *N. maculata*, as has been pointed out for this and other Hemiptera species (3).

Our results seem to show : absence of differential selectivity for the three prey species studied; higher response percentage and lower response time to live prey ahead of dead ones (only not statistically significant for response to bees); no clear size selection within the same prey species (probably because of small differences) and a higher response percentage and lower response time to vibrations with higher frequencies (representing a possible prey).

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Preliminary data on the genetic differentiation of populations of three frog species (Anura, Amphibia) from Cyprus and Greece

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Electrophoretic analysis was carried out on populations of *Rana ridibunda*, *Hyla savignyi* and *Bufo viridis* of Cyprus and compared with data from populations from Greece in an attempt to study their genetic variation. Enzymes and other proteins were separated using standard starch and polyacrylamide gel electrophoresis. Electrophoretic conditions were as described in our previous studies (1) (2) (3) and data analysed using the BIOSYS-1 computer package (4). Levene's (5) correction for small sample size was employed in chi-square analyses. The phylogenetic structure of the population was analysed with CONTML (PHYLIP 3.57c package) (6) based on allele frequencies. The loci, alleles and allele frequencies, degree of heterozygosity (H) and proportion of polymorphic loci (P) found in all Cypriot and Greek populations are shown in Table 1 (in appendix).

***Rana ridibunda* :** The eight enzyme and two protein systems investigated encode fifteen presumptive genetic loci. Many polymorphic loci had genotype frequencies not in good agreement with Hardy-Weinberg expectations (chi-square, $P < 0.05$). This may reflect mixing of different populations or sampling error. *R. ridibunda* populations from Greece and Cyprus differ mainly in two loci, LDH-2 and MProt. The phylogenetic analysis (Fig.1) showed two separate groups. *Rana epeirotica* (7) and *Rana cretensis* (8) form one group and *R. ridibunda* and *Rana balcanica* (9) the other one. The Cypriot *R. ridibunda* is closest to the Greek *R. balcanica.*

This tree is in agreement with a UPGMA tree constructed based on genetic identity values (not shown). From the allelic frequencies at the 15 loci tested, we calculated Nei's (10) values of genetic identity (I) and genetic distance (D). The values of D (0.202 and 0.223 respectively), found between both Cypriot populations and the Greek *R. ridibunda*, indicate that these populations are separated. On the other hand, the values of D (0.022 - 0.034, mean=0.028) between the Cypriot *R. ridibunda* and the Greek *R. balcanica* indicate that these populations are probably conspecifics. Eventually, the values of D between Cypriot *R. ridibunda* and both other species, *R. epeirotica* (D=1.152*)* and *R. cretensis* (D=1.023), demonstrate a higher differentiation. From all the above data we suggest that the water frogs of Cyprus are related to the Greek *R. balcanica*. The high degree of polymorphism found only in the Evros population has also been reported in other studies (1) (11). This region probably represents a hybrid zone between the Greek *R. ridibunda* and *Rana bedriagae* of Anatolia, and gene flow occurs between them. Further studies including populations of *R. bedriagae* from Anatolia would provide more accurate data for a more final conclusion.

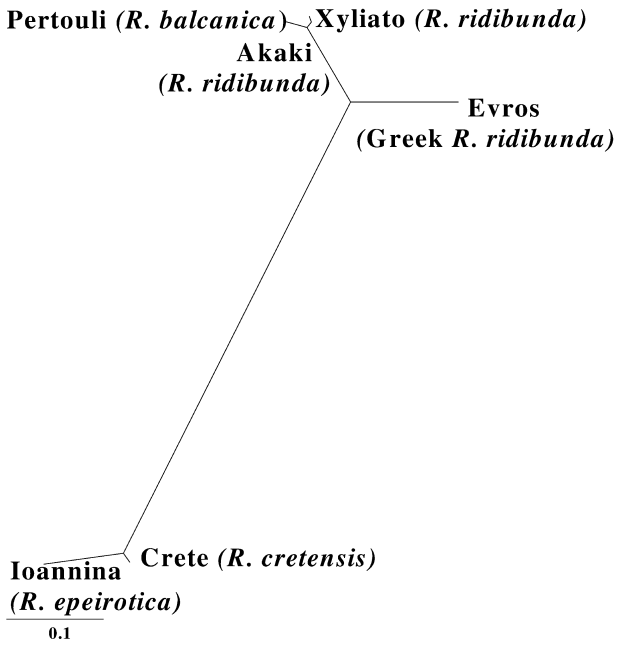

Fig. 1. – Maximum likelihood tree (CONTML, Felsenstein 1993) based on the allele frequencies of each population of water

***Hyla savignyi* :** The five enzyme and two protein systems investigated encode ten presumptive genetic loci. Most polymorphic loci had genotype frequencies in good agreement with Hardy-Weinberg expectations (chi-square, $P > 0.05$). Information on genetic structure of *H. savignyi* is very limited. A lower average heterozygosity (0.088) has been reported for *H. arborea savignyi* in Israel (12). The values of genetic identity (I) between the two populations of *H. savignyi* from Cyprus were 0.992 and between *H. savignyi* and Greek *H. arborea* 0.698 while values of D were 0.008 and 0.366 respectively. These results indicate that populations of *H. savignyi* of Askas and Sotira are the most closely related and differ substantially from the population of *H. arborea* from Greece. The high degree of genetic identity is typical in island populations (13).

***Bufo viridis* :** The five enzyme and two protein systems investigated encode twelve presumptive genetic loci. Populations of *B. viridis* from Greece and Cyprus mostly

differ in two loci (ALB-1 and Hb-1). Most polymorphic loci had genotype frequencies in good agreement with Hardy-Weinberg expectations (chi-square, $P > 0.05$). Values of D and I between the two studied populations were 0.740 and 0.301 respectively. All genetic parameters showed that the population of *B. viridis* from Cyprus is more polymorphic than the Greek one. The results of the present study clearly showed that the populations of *B. viridis* from Cyprus and Greece are greatly differentiated.

Concluding this paper we have to underline that the studied frog species of Cyprus are genetically differentiated from the Greek ones. However, our data are preliminary and more detailed studies using additional methods, are needed, in order to elucidate the taxonomic status of these species and to give a global approach.

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APPENDIX : TABLE 1

Loci, alleles and allele frequencies, degree of heterozygosity (H), proportion of polymorphic loci (P) mean alleles per locus (M.N.A) found in frog species from Cyprus and Greece

Taxon		***R.ridibunda***		***R. balcanica***	***R. ridibunda***	***R. epeirotica***	***R. cretensis***	***H. savignyi***		***H. arborea***	***B. viridis***	
		Cyprus		**Greece**				**Cyprus**		**Greece**	**Cyprus**	**Greece**
Locality		**Xyliato**	**Akaki**	**Pertouli**	**Evros**	**Ioanninaa**	**Cretea**	**Askas**	**Sotira**	**Komotini**	**Ag. Dometios**	**Evros**
n		**25**	**7**	**6**	**10**	**5**	**4**	**9**	**34**	**9**	**8**	**5**
Locus	Allele											
AAT-1	A	1.00	1.00	1.00	1.00	1.00	1.00	0.88	0.76	1.00	1.00	1.00
	B	-	-	-	-	-	-	0.11	0.23	-	-	-
AAT-2	A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ADH-1	A	1.00	1.00	1.00	0.40	-	-	-	-	-	-	-
	B	-	-	-	-	1.00	1.00	-	-	-	-	-
	C	-	-	-	0.30	-	-	-	-	-	-	
	D	-	-	-	0.20	-	-	-	-	-	-	-
	E	-	-	-	0.10	-	-	-	-	-	-	-
CK-1	A	1.00	1.00	1.00	0.40	-	-	1.00	1.00	1.00	1.00	1.00
	B	-	-	-	-	1.00	1.00	-	-	-	-	-
	C	-	-	-	0.60	-	-	-	-	-	-	-
EST-1	A	0.50	0.50	0.50	-	-	-	-	0.10	0.11	0.75	0.30
	B	0.50	0.50	0.50	-	-	-	0.27	-	0.33	0.25	0.70
	C	-	-	-	0.05	-	-	0.72	0.80	0.22	-	-
	D	-	-	-	0.80	-	-	-	0.08	0.33	-	-
	E	-	-	-	0.10	-	-	-	-	-	-	-
	F	-	-	-	0.05	-	-	-	-		-	-
EST-5	A	1.00	1.00	1.00	0.80	-	-	-	-	1.00	0.56	0.20
	B	-	-	-	0.20	-	-	1.00	1.00	-	0.44	-
	C	-	-	-	-	-	-	-	-	-	-	0.80
GPD-1	A	1.00	1.00	1.00	0.40	-	-	-	-	-	-	-
	B	-	-	-	-	1.00	1.00	-	-	-	-	-
	C	-	-	-	0.60	-	-					
LDH-1	A	1.00	1.00	1.00	0.40	-	-	0.16	0.25	0.50	0.31	-
	B	-	-	-	-	1.00	1.00	0.83	0.75	0.50	-	0.50
	C	-	-	-	0.30	-	-	-	-	-	0.69	0.50
	D	-	-	-	0.20	-	-	-	-	-	-	-
	E	-	-	-	0.10	-	-	-	-	-	-	-
LDH-2	A	0.64	0.93	1.00	1.00	1.00	1.00	-	-	-	1.00	1.00
	B	0.36	0.07	-	-	-	-	-	-	-	-	-
MDH-1	A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.69	-
	B	-	-	-	-	-	-	-	-	-	-	0.50
	C	-	-	-	-	-	-	-	-	-	0.31	0.50
MDH-2	A	1.00	1.00	1.00	1.00	-	-	-	-	0.61	0.44	0.20
	B	-	-	-	-	1.00	1.00	1.00	1.00	0.39	0.56	0.80
PGM-1	A	1.00	1.00	1.00	0.40	-	-	-	-	-	-	-
	B	-	-	-	-	1.00	1.00	-	-	-	-	-
	C	-	-	-	0.60	-	-	-	-	-	-	-
PGM-2	A	1.00	1.00	1.00	0.40	0.00	0.00	-	-	-	-	-
	B	-	-	-	-	1.00	1.00	-	-	-	-	-
	C	-	-	-	0.60	-	-	-	-	-	-	-
Hb-1	A	0.50	0.50	0.50	0.50	-	-	-	-	0.50	0.50	-
	B	0.50	0.50	0.50	0.50	-	-	1.00	1.00	0.50	0.50	1.00
ALB-1	A	-	-	-	-	-	-	-	-	0.50	0.44	-
	B	-	-	-	-	-	-	0.83	0.80	-	0.56	-
	C	-	-	-	-	-	-	0.80	0.19	0.50	-	1.00
ALB-2	A	-	-	-	-	-	-	-	-	-	0.94	0.70
	B	-	-	-	-	-	-	-	-	-	0.06	0.30
MProt	A	1.00	1.00	0.50	1.00	-	0.50	-	-	-	-	-
	B	-	-	0.50	-	-	0.50	-	-	-	-	-
	C	-	-	-	-	0.50	-	-	-	-	-	-
	D	-	-	-	-	0.50	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-
H		0.09	0.08	0.11	0.31	0.04	0.04	0.12	0.13	0.28	0.34	0.27
P		20.0	20.0	20.0	60.0	8.33	8.33	40	40	50	75.00	58.33
M.N.A.		1.20	1.20	1.20	2.07	1.08	1.08	1.40	1.50	1.70	1.75	1.58

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The Multicoloured Asian Ladybird *Harmonia axyridis* Pallas (Coleoptera : Coccinellidae), a threat for native aphid predators in Belgium ?

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KEY WORDS. Multicoloured Asian Ladybird, *Harmonia axyridis*, invasive alien species, biological control, Belgium.

The Multicoloured Asian Ladybird (*Harmonia axyridis* Pallas 1773) is native to large parts of Asia. It preys mostly on tree-dwelling hemipteran insects such as psyllids, scale insects and aphids (1). It is a commercially attractive biocontrol agent for aphid populations because its larvae are very voracious, polyphagous and easy to rear (2-4). *H. axyridis* is widely used for reducing pest aphid populations in greenhouses, orchards and gardens in North America since 1916 and in Western Europe since 1982, where it is sold by different private companies (e.g. Biotop SAS, BioBest and Koppert) (1,4). Little attention has been paid to the development of feral populations of *H. axyridis* in Europe. This is surprising given the rapid colonisation of a wide range of American ecosystems, and growing concerns over the negative impact of natural enemy introductions (5-7). Recent observations suggest that this species is now invading (semi-)natural ecosystems in Belgium and may become a potential threat for native ladybird species and other aphid predators.

Adults of *H. axyridis* are strongly oval and convex in shape, measure 5-8 mm and are larger than most of the indigenous ladybird species. The elytra usually display a wide "keel" at the apex. They are highly colour polymorphic with elytra ranging from pale yellow-orange to black bearing 0-19 spots. The head, antennae, and mouthparts are generally straw-yellow but are sometimes tinged with black. The pronotum is similarly straw-yellow with up to five black spots or with lateral spots usually joined to form two curved lines, an M-shaped mark, or a solid trapezoid.

Larvae are elongate, somewhat flattened, and adorned with strong tubercles and spines. The mature larva is distinctively and strikingly coloured. The overall ground colour is mostly black to dark bluish-grey, with a prominent bright yellow-orange patch extending over the dorsolateral lobes of abdominal segments 1-5 on each side. A more detailed description of the species is given by (1).

A large scale field survey of ladybirds was launched in Belgium in 1999 by the *Coccinula* working group, initiated by "Jeunes & Nature" and the Research Centre for Nature, Forests and Wood in the Walloon Region and the "Jeugdbond voor Natuurstudie en Milieubescherming" and the Institute of Nature Conservation in the Flemish Region. The number of collaborators increased rapidly (about 450 at present) and a large part of the Belgian territory is now being surveyed for ladybirds.

Although *H. axyridis* has been used for biological control in Belgium since 1997 (8), no observations in the wild were reported until September 2001 (Ghent). Since then, the number of observations increased steadily (Fig. 1), especially in the provinces of Brabant and Antwerp (Fig. 2).

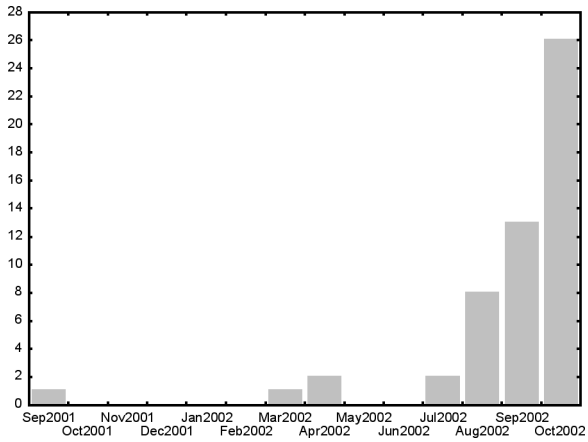

Fig. 1. – Trend in the number of field observations of *Harmonia axyridis* until october 2002.

Although numerous observations originated from cities and anthropogenic sites, individuals were also found in (semi-)natural habitats such as forests and meadows (e.g. Wilrijk, Houwaart, Olen and Koersel). Most individuals were found on deciduous trees, especially lime (*Tilia* sp.) and maple (*Acer* sp.). Observations of eggs, larvae and pupae are now widespread and demonstrate that the Multicoloured Asian Ladybird reproduces successfully in many places. Overwintering aggregations were also observed in houses (e.g., Brussels and Antwerp) since the end of October 2002. Observations of feral populations elsewhere in Europe have recently been reported in Greece (9), Southern France (4,10), and Germany (11).

Studies of the expansion of *H. axyridis* in North America showed that it can rapidly colonise large areas. After some time, this species often becomes the

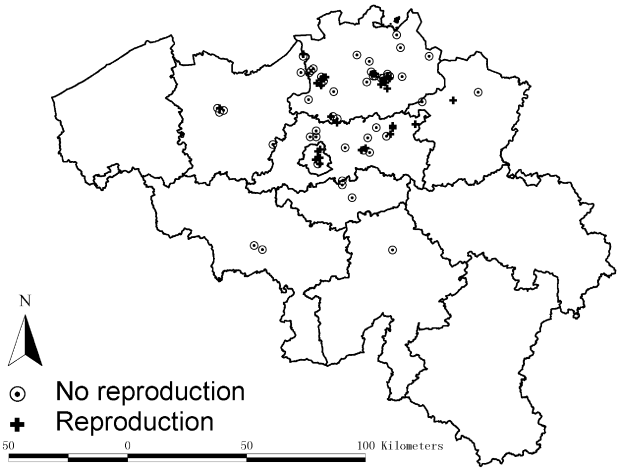

Fig. 2. – Sites with observations of *Harmonia axyridis* since September 2001. Reproduction is indicated when eggs, larvae and/or pupae were observed.

predominant species in aphidophagous guilds and can induce the decline of native ladybird species (12-14). Furthermore, it is a very good colonizer and is very competitive because it :

- has a wide trophic niche and a high level of phenotypic plasticity for several life-history traits (15),
- is very voracious and is often involved in intraguild interactions with other aphidophagous species, including *Adalia bipunctata* (L.) and *Coccinella septempunctata* L., two ladybird species native to Western Europe. In this case, it frequently behaves as an intraguild predator (16-18),
- has strong dispersal capacities and undertakes long range migrations to overwintering sites (19-21).

If the exponential increase in the number of *H. axyridis* populations continues in following years, it is likely that it will invade most of the (semi-)natural ecosystems in Belgium (and in the neighbouring countries), causing harm to native aphidophagous species. In this context, the responsibility of private companies (selling *H. axyridis* or other biocontrol agents) should be pointed out. The example of *H. axyridis* demonstrates the urgent need for risk assessment procedures before biocontrol agents can be sold and spread on a large scale, as is already imposed for environmental dissemination of pesticides and genetically modified organisms. Furthermore, given the example of N-America, studies on feral populations of *H. axyridis* in Belgium, and other affected countries, are urgently needed in order to estimate its impact on the indigenous aphidophagous fauna.

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